"The Use of Dynamic Culture Devices in Articular Cartilage Tissue Engineering"

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Abstract

Tissue engineered repair of articular cartilage has now become a clinical reality with techniques for cell culture having advanced from laboratory experimentation to clinical application. Despite the advances in the use of this technology in clinical applications, the basic cell culture techniques for autologous chondrocytes are still based on primitive in-vitro monolayer culture methods.

Articular chondrocytes are known to undergo fibroblastic change in monolayer culture as this is not their normal state in-vivo. They are more likely to maintain their phenotype when cultured in three dimensional environments. In this state they become spherical in shape and synthesise normal cartilage matrix products. Various substances are being presently investigated with the aim of designing a suitable material that is biocompatible, biodegradable and suitable for implantation. The major problem of culturing cells in three dimensional scaffolds is the limitation posed by the biomaterial on nutrient diffusion to cells deep within the scaffold. In order for this technology to succeed in clinical practice there is a important need to develop solutions to overcome these diffusional restraints. The use of dynamic culture devices which can, not only stimulate chondrocytes, but also maintain their original characteristics are investigated in this project.

This thesis tests the hypothesis that culture within a dynamic culture device ie a rotating wall vessel bioreactor or roller bottles, enhances proliferation and cartilage-specific matrix synthesis by chondrocytes seeded in a three dimensional construct. The long term survival of chondrocytes in hydrogel matrices is also examined and cell cultures in dynamic devices are compared with traditional static culture systems. Biochemical, histological and immunostain data is presented an the possibility of using human cells is also explored.

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"Nothing great was ever achieved without enthusiasm."
Ralph Waldo Emerson

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Thesis Statement

Millions of people worldwide suffer some type of articular cartilage damage with causes ranging from mechanical trauma to degenerative diseases such as osteoarthritis. The health care cost associated with cartilage defects has spurred research to alleviate this common and burdensome problem. Articular cartilage, unlike bone, exhibits a limited ability to repair itself *in vivo*. Mechanical injuries that result in a tear or abrasion of the articular cartilage will generally undergo repair with fibrous tissue which does not adequately tolerate weight bearing and thus unable to effectively withstand continuous physiological loads leading possibly to early joint degeneration.

Tissue engineered repair of articular cartilage has now become a clinical reality with techniques for cell culture having advanced from laboratory experimentation to clinical application. The technique of Autologous Chondrocyte Implantation (ACI) has been a direct product of close collaboration between scientists and clinicians. The early results from ACI have been extremely encouraging. However, this field is relatively new and improvement and refinement of the methods ie laboratory culture techniques, instruments for performing the surgery, methods of assessing outcome and methods for assessing quality of repair tissue, will no doubt occur in the near future.

Attempts to repair cartilage with the use of transplants such as autografts have met with limited success. Alternative treatments have arisen from the use of tissue engineering strategies. Recently, biodegradable polymer matrices seeded with chondrocytes have been investigated as alternatives for cartilage replacement. Previous studies have focused on seeding constructs with chondrocytes and observing growth under static conditions. The cell yields were poor and quality of matrix produced was unsuitable for clinical application using these static culture systems. More recent data demonstrate that constructs grown in spinner flasks resulted in production of more glycosaminoglycan (GAG) and collagen than their static counterparts. Despite these encouraging results, their still lacks a quantitative evaluation of cartilage construct growth in a flow environment.

The potential of improving cell phenotype by culturing in three dimensional scaffolds has been the focus of interest for numerous tissue engineering research groups. The difficulties underlying the successful transfer of these laboratory techniques to clinical application are based on the successful survival of cells within constructs made of various biocompatible materials. Cells are extremely sensitive to their environments and in particular have an important need for consistent nutrient transfer and removal of waste products. The limitation posed by most biomaterials used in tissue engineering to these important factors makes them impractical for use in culturing cells because they are not able to provide sufficient cell yields for the purpose of clinical implantation.

The National Institute of Clinical Excellence has made key recommendations on the use of autologous chondrocyte implantation in a clinical setting. Following a comprehensive review, they have concluded that the effectiveness of the procedure needs further assessment. There is a strong obligation on orthopaedic specialists to deal with issues concerning clinical outcomes and assessing quality of repair for the ACI procedure. The need for fully investigating these techniques in a laboratory setting and understanding the mechanisms and processes by which they work and also investigating methods of improvement are of significant relevance.

The availability of facilities for advanced culture techniques, clinical experience and laboratory set up was available at the Institute of Orthopaedics and it offered me a unique opportunity to perform detailed studies on improving the current methods of articular chondrocyte culture. Autologous chondrocyte implantation, presently in its infancy, hopes to benefit hundreds of patients in the future and therefore the need to enhance cell culture techniques for this procedure are vital in order to ensure safety and longevity of the procedure for patients in the future. This research investigation explores the possibilities of enhancing this cartilage repair technology using advanced culture techniques.

Chapter I

Introduction

1.1 Aims & Objectives

The aim of this thesis was to investigate methods of improving existing tissue engineering systems for repair of articular cartilage defects by the use of dynamic culture devices. The initial part of the project was aimed at finalising studies on the use of a novel collagen/alginate sponge for cartilage repair that had been commenced by my predecessor. In particular there was a need to investigate its ability to function mechanically as a construct in a dynamic culture environment. Previous work on the use of dynamic culture devices was limited and therefore it was the intention of the project to determine the usefulness of these devices in the tissue engineering of articular cartilage.

The internal meshwork architecture of the collagen sponge to some extent resembles the internal architecture of collagen fibrils within normal articular cartilage. It not only supports chondrocyte culture but it may also help to improve matrix integration. Unfortunately its major drawback is that it is mechanically weak and easily deforms under pressure. Although it may support chondrocytes biochemically, mechanically it only offers chondrocytes an attachment surface and does not provide chondrocytes a true three dimensional environment for reception and transmission of mechanotransduction signals. Locally produced growth factors and hormones are easily washed away due to the very large pore sizes.

It was therefore proposed that using alginate as a filling material would provide a satisfactory solution to the above problems. Alginate also supports chondrocytes and has been shown to perform remarkably well on its own in terms of supporting chondrocyte growth and matrix production. It provides cells with a true three dimensional environment and therefore may allow transduction of mechanical signals and hormonal signals. The collagen/alginate construct, in theory, appeared very attractive and therefore was investigated by my predecessor. The production of a collagen/alginate construct containing chondrocytes was successful. It was found that the best method of incorporating the alginate into the collagen sponge was to allow a

gradual absorption of alginate into the collagen sponge while the alginate was in liquid form. It was found to support chondrocyte cultures successfully in short term cultures. However it was discovered that although successful in maintaining chondrocyte growth and matrix production, it too, like other scaffold systems, suffered from causing cell death at the centre of the construct. Previous literature has confirmed that the most likely cause for this was diffusional constraints on nutrients. Its poor mechanical strength was also seen to be a disadvantage, however it was thought that by improving nutrient diffusion and allowing a longer term for culture there would be improved mechanical strength of the construct due to the newly synthesised extracellular matrix which would offer support.

The **first objective** of this thesis was to investigate the physical properties of the collagen/alginate construct and propose methods of improving the design. Experiments to assess the expansion properties of the collagen and alginate components of the scaffolds were investigated.

The **second objective** of this thesis was to investigate the use of methods of improving mass transfer of nutrients into scaffolds. Roller bottles allow a continuous circulation of medium and it was postulated that they would help to enhance the diffusion of medium into the constructs and thereby improve mass transfer of nutrients into the centrally located chondrocytes. As all previous experiments had been performed on static culture dishes, it was thought there was a poor diffusion gradient developing across the surface of these constructs which resulted in poor mass transfer of nutrients. Experiments using roller bottles for culturing chondrocytes embedded within collagen/alginate constructs were performed. Histological examination of the constructs was performed.

Following analysis of results from the study on roller bottles a more advanced dynamic culture system was investigated. The rotating wall vessel bioreactor has previously been used in cell cultures. Although its use for chondrocyte cultures had been very limited it nevertheless satisfied the criteria for use in our experiments.

The **third objective** of my thesis was to investigate the use of the rotating wall vessel device as a model bioreactor in the culturing of chondrocytes embedded in three dimensional scaffolds. As the collagen/alginate construct from my initial experiments showed considerable limitations it was necessary to use agarose as the construct

biomaterial. The use of agarose allowed a more direct comparison to be made with other culture systems using agarose. The initial work commenced on use of disc constructs.Later agarose bead constructs which were of smaller dimensions than discs were used.

The successful results using the bioreactor model prompted further investigation of the potential of using these culture techniques on a different biomaterial composed of alginate. Alginate is a biocompatible material and has more relevance for clinical use than the agarose model. It offers advantages in that it can be dissolved easily and the chondrocytes may be retrieved for serial passage. Alginate constructs containing chondrocytes have already been used in human trials. The **fourth objective** was to investigate the use of alginate constructs as chondrocyte scaffolds cultured in the same conditions as previously investigated using the dynamic culture device. Particular emphasis was placed on chondrocyte characterisation and matrix analysis to ensure that the chondrocyte phenotype was maintained. Experiments using alginate beads were performed. Results were analysed using histological, biochemical and other more advanced characterisation techniques.

The **final objective** of the thesis was to investigate the potential of using human rather than bovine chondrocytes in the above bioreactor system. It has been reported that human chondrocytes are less responsive to growth and matrix production than other species and therefore the ultimate challenge for tissue engineering scientists would be to successfully culture and multiply human chondrocytes.

Chapter II

Literature Review

2.1 Articular cartilage structure and Properties

2.1.1 Overview

Articular cartilage, the resilient load-bearing material of diarthrodial joints, provides joints with excellent friction, lubrication, and wear characteristics required for continuous gliding motion. It also serves to absorb shock and distribute the applied load uniformly to the underlying bony supporting structures. Although remaining biomechanically stable under normal physiological conditions over seven to eight decades of life it can undergo progressive degradation and destruction if it becomes damaged by either direct or indirect trauma or by degenerative joint disease. Often, total joint replacement is the only treatment of choice to relieve the considerable morbidity and to restore pain free mobility to the affected joint. Recent advances in the understanding of articular cartilage biology, composition, metabolism, molecular and ultrastructural organisations, and biomechanical properties offers hope for the development of biologically based repair procedures as alternatives to prosthetic joint replacements in the treatment of early damage and/or degenerative joint diseases. The goal of generating a viable substitute for articular cartilage through tissue engineering concepts now appears to be achievable in the near future. This chapter presents a review of the current understanding of articular cartilage structure and function in health and disease and forms a basis for the experimental work undertaken in this thesis.

2.1.2 Introduction

Hyaline cartilage with its characteristic partially translucent glass (Greek, Hyalos) like appearance derives its characteristics from components of the extracellular matrix. It has been present in vertebrate organisms since their origin more than a half-billion years

ago. Other forms of cartilage also found in vertebrates include fibrous cartilage (intervertebral disks, menisci, repair cartilage) and elastic cartilage (ear, nose, trachea and rib cage) (Stockwell 1978b). Articular cartilage is devoid of nerves and is generally considered to be avascular, although a few blood vessels may be found in its deepest parts adjacent to bone. Sparse in its cell content (<10% of tissue) it contains an abundance of extracellular matrix (Stockwell 1978a) composed of water (60-80%), collagen (20%), proteoglycans (5-10%), non-collagenous proteins (< 5%) (Maroudas 1972)and small amounts of lipid and inorganic chemicals. In articular cartilage, although chondrocytes organise the collagens, proteoglycans, and non collagenous proteins into a unique, highly ordered structure, the thickness, cellularity and other morphological, chemical and physical properties of articular cartilage show biological variation between subjects. Differences may also be identified between species and even different sites in the same subject. Cartilage derives its nutrition from synovial fluid, which is freely transported across the articular surface during normal joint loading.

2.1.3 Structure of Articular Cartilage

Articular cartilage varies in its nature according to depth from the articular surface (Buckwalter & Mankin 1998b). The changes in chondrocyte and matrix morphology from the surface to the subchondral bone make it possible to identify four layers, or zones. These consist of a superficial zone, a transitional (middle) zone, a deep zone and a zone of calcified cartilage. Both between and within species, these zones differ morphologically with their boundaries being difficult to identify. Within individual zones, distinct matrix regions can be identified, including the pericellular matrix, territorial matrix and the interterritorial matrix (Poole, Flint, & Beaumont 1984).

Zones of articular cartilage

The *superficial zone* is the thinnest of the zones (40μm) and forms a gliding surface. The thin collagen fibrils are arranged parallel to the surface which give this zone greater stiffness and strength than the deeper zones, and they may resist shear forces generated during use of the joint. The chondrocytes appear elongated and form ellipses with their long axes arranged parallel to the surface. The surface 2μm layer (Lamina splendans) of the superficial zone is composed of fine densely packed collagen fibrils and is non-cellular (Clark 1990). The superficial zone makes an important contribution to the

compressive behavior of articular cartilage (Setton et al. 1993). Disruption or remodeling of the dense collagenous matrix of the superficial zone is one of the first detectable structural changes in experimentally induced degeneration of articular cartilage, suggesting that alterations in this zone may contribute to the development of osteoarthrosis by changing the mechanical behavior of the tissue (Buckwalter & Mankin 1998d). Removal of this zone is found to increase the permeability of the tissue and probably increases loading of the macromolecular framework during compression.

The *transitional or middle zone* is a much thicker zone of approximately 500μm. The chondrocytes are more spherical and their enlarged cytoplasm is full of endoplasmic reticulum and Golgi bodies. The collagen fibrils are larger in diameter and more randomly orientated (Jeffery et al. 1991).

The *deep zone* is the thickest of all zones (>500μm). The chondrocytes are also spherical in this zone and they are arranged in perpendicular columns of up to nine cells. The zone contains the largest collagen fibrils that also have a perpendicular arrangement. The proteoglycan content is higher than the more superficial zones although the associated aggregates are smaller leading to a lower water content in this zone.

The thin zone of calcified cartilage separates the soft hyaline cartilage from the stiffer subchondral bone. The chondrocytes are small and spherical with very little cytoplasm and endoplasmic reticulum in their cytoplasm suggesting that they have a very low metabolic activity. Collagen fibrils from the deep zone penetrate the zone of calcified cartilage and anchor the cartilage to subchondral bone. A thin basophilic line seen on light microscopy known as the 'tidemark' corresponds to the boundary between calcified and uncalcified cartilage. The zone of calcified cartilage acts as a barrier blocking free nutrient and metabolite diffusion from and to the subchondral bone (Muehleman & Arsenis 1995).

Matrix regions of articular cartilage

The extracellular matrix of articular cartilage is non-uniform in distribution. It contains distinct regions of varying collagen and proteoglycan content. Three zones of matrix may be identified around individual chondrocytes; a pericellular region and a territorial

region which appear to protect the cells from loading and deformation of the tissue and may also help to transmit mechanical signals to the chondrocytes when the matrix deforms during joint-loading. The interterritorial region, which surrounds the pericellular and territorial regions, functions to provide the bulk of the mechanical strength to the cartilage (Hunziker, Michel, & Studer 1997).

The *pericellular matrix* is a thin shell which encloses and is attached to the chondrocyte cell membrane. It has little or no fibrillar collagen but is rich in proteoglycans and also contains non-collagenous proteins such as anchorin CII (Mollenhauer et al. 1984). Cytoplasmic extensions from the chondrocytes project into and through the pericellular matrix to the territorial matrix.

The *territorial matrix* surrounds the pericellular matrix of individual or clusters of chondrocytes. Collagen fibrils within the territorial matrix appear to adhere to the closely pericellular matrix whereas fibrils located at a distance from the cell decussate and intersect to form a fibrillar basket which may be responsible for the mechanical protection of the chondrocytes during loading and deformation of the tissue. The territorial –interterritorial boundary is marked by an abrupt increase in the diameter of the collagen fibrils that become more parallel in alignment.

The *interterritorial matrix* contains the largest diameter collagen fibrils and makes up most of the volume of mature articular cartilage. The collagen fibrils within the interterritorial matrix are arranged according to their organisation as discussed earlier.

2.1.4 Composition of Articular Cartilage

2.1.4.1 Chondrocytes

Within normal articular cartilage there is only one type of cell, the highly specialised chondrocyte (Buckwalter & Mankin 1998b). These cells contribute little to the volume of the tissue, less than 10% in adult human cartilage (in species, especially small animals such as mice, rats, and rabbits, which have thin articular cartilage, the cell density is many times greater than in humans (Stockwell 1978a;Stockwell 1967). Chondrocytes from different cartilage zones differ in size, shape, and probably

metabolic activity (Aydelotte, Greenhill, & Kuettner 1988), but all of these cells contain organelles required for matrix synthesis, including endoplasmic reticulum and Golgi membranes. They also frequently contain intracytoplasmic filaments, lipid, glycogen, and secretory vesicles, and at least some chondrocytes have short cilia extending from the cell into the matrix. These structures may have a role in sensing mechanical changes in the matrix. Although the total metabolic activity of cartilage is low, individual cells have a high glycolytic rate per cell similar to that of cells in vascularised tissues (Buckwalter & Mankin 1998b). A spheroidal shape distinguishes chondrocytes from other cells. Chondrocytes are able to respond to a variety of environmental stimuli. These stimuli include soluble mediators, such as growth factors, interleukins, and pharmaceutical agents; matrix composition; mechanical loads; and hydrostatic pressure changes. Chondrocytes are responsible for the synthesis of appropriate types and amounts of macromolecules that are required to maintain the articular surface despite the constantly changing macromolecular framework of the matrix in response to use of the joint. As discussed earlier, chondrocytes may actually possess the ability to sense changes in the cartilage environment. By producing enzymes, chondrocytes may degrade the matrix and then, by increasing synthetic activity, replace the degraded components. The mechanisms responsible for the regulation of this turnover are poorly understood. It is however, well established that mechanical effects play an important role in chondrocytic activity, the frequency and intensity of joint loading for example influences chondrocyte activity, in joint loading degradation of matrix exceeds synthesis whereas in immobilisation, the reverse occurs. It may therefore be through a process of mechano-transduction that chondrocytes sense environmental changes.

In adult animals, chondrocytes derive their nutrition from nutrients in the synovial fluid, which, to reach the cell, must pass through a double diffusion barrier, involving both the synovial tissue and synovial fluid, and then the cartilage matrix. This latter barrier is restrictive not only with respect to the size of the molecules but also with respect to other features such as charge and molecular configuration (Fischer et al 1995).

Throughout skeletal growth, the activity and function of chondrocytes changes. In growing individuals, chondrocytes produce new tissue to expand and remodel the articular surface, by contrast, in skeletally mature individuals they do not substantially change the volume of the tissue, but they replace degraded matrix molecules and they may remodel the articular surface. With ageing, the capacity of the cells to synthesise

some types of proteoglycans and their response to stimuli, including growth factors, decrease (Buckwalter, Roughley, & Rosenberg 1994).

2.1.4.2 Extracellular Matrix (ECM)

The matrix of articular cartilage may be considered to be biphasic in nature. It consists of a liquid phase (tissue fluid) composed of water, dissolved gases, small proteins, metabolites, and a high concentration of cations to balance the negatively charged proteoglycans and a solid phase (structural macromolecules) composed of collagen, proteoglycans and non collagenous proteins (Mow, Holmes, & Lai 1984).

Structural Macromolecules

The structural macromolecules of cartilage, namely collagens, proteoglycans, and non-collagenous proteins, contribute 20% to 40% of the wet weight of the tissue. They vary in concentration within the tissue and also make different contributions to the overall tissue properties.

Collagens

Collagens are the most abundant proteins in mammals and constitute up to one quarter of the body weight. They contribute about 60% of the dry weight of cartilage. To date, nineteen types of collagen have been identified which have been encoded by more than thirty genes. Although they are more abundant in the superficial zone, collagens are distributed relatively uniformly throughout the depth of the cartilage.

The collagen forms a three-dimensional meshwork of small diameter fibrils within which aggregates of proteoglycans are entrapped. The resiliency and tensile strength of cartilage is considered to arise from the restraining effect of collagen fibrils on these aggregates (Irwin & Mayne 1986). By definition a collagen must have a structural function in extracellular matrix and must incorporate a triple helix structure in which three left handed helices (α-chains) twist around each other to form a right handed superhelix (Thomas, Ayad, & Grant 1994). The high proline content (about 25% of total residues) causes each chain to exhibit the characteristic left-handed helical configuration. The triple helix conformation is the result of the α-chain polypeptides

incorporating an unusual repetitive amino acid sequence of a glycine residue (about 33% of total content) i.e. $(Gly\text{-}X\text{-}Y)_n$. Glycine, the smallest of the amino acids, participates in the hydrogen bonding between the three strands of the triple helix.

Hydroxyproline, hydoxylysine, and glycosylated hydroxylysine form most of the remaining residues of the repeating sequence. Hydroxyproline through its intramolecular hydrogen bonding maintains the helical conformation. Hydroxylysine stabilises collagen supramolecular aggregates (Cima et al. 1981;Eyre, Wu, & Apone 1987). Ascorbate has recently been shown to influence synthesis of collagen and deficiency of ascorbate in vitro culture conditions leads to under hydroxylation of the collagen molecules leading to an unstable helical configuration (Ronziere et al. 1997).

Articular cartilage contains collagen types II, VI, IX, X and XI. Types II, IX and XI form cross-banded fibrils seen with electron microscopy. These cross bands are a result of the alignment of the collagen monomers and are typical for type II collagen. Types IX and XI form thinner fibrils and may act as nuclei for the deposition of type II collagen.

Type II collagen is the major type of collagen found in articular cartilage and it represents about 90-95% of the total collagen content of adult cartilage accounting for the primary component of the cross-banded fibrils. It is a homotrimer with three identical α-chains. The COL2A1 gene codes for type II procollagen which may be expressed in two different forms (Type IIA and type IIB) depending on the differential splicing of the gene. Type IIB mRNA transcript is expressed by chondrocytes in cartilaginous tissue while type IIA is expressed by mesenchymal or fibroblast like cells in prechondrogenic regions surrounding developing cartilage (Ryan, Sieraski, & Sandell 1990). Type XI collagen molecules bind covalently to type II collagen molecules and probably form part of the interior structure of the cross-banded fibrils. The function of type XI collagen is uncertain but there is evidence that it may play an important role in the regulation of the diameter of the fibrils (Lui et al. 1995).

Type VI collagen is a heterotrimer with three distinct α-chains. It represents about 2% of the total collagen content of adult articular cartilage. It lacks the lysine-derived crosslinks and instead contains disulphide bridges. It is believed that type VI collagen has a role in binding cells to their extracellular matrix.

Type IX collagen is also a heterotrimer with three distinct α-chains encoded by separate genes. It represents approximately 1-2% of the total collagen content of adult articular cartilage. It is a non-fibrillar collagen containing non-collagenous domains and biochemically exists in two forms, one of which may be classified as a proteoglycan (Ayad et al. 1991). Type IX collagen forms covalent hydroxylysine-derived cross-links with type II collagen (van der Rest & Mayne 1988)and under electron microscopy appears to coat the surface of the collagen type II fibrils (Eyre, Wu, & Woods 1998).

Type X collagen is a short homotrimer, which consists of a single triple helix and is found in endochondral ossification, such as the hypertrophic zone of the growth plate of animals and the calcified zone of mature articular cartilage (van der Rest and Garrone 1991a). It has been shown that collagen type X forms a scaffold like structure within the pericellular matrix which may act as a marker for targeting components in the degradation process and possibly replacement of cartilage by bone (Buckwalter & Mankin 1998a). Morrison et al (1995) (Morrison et al. 1996a) have also demonstrated the synthesis of collagen type X in hypertrophic chondrocytes by immunolocalisation.

Type I collagen is the most abundant fibrillar collagen in the connective tissues of the body and it is found mainly in skin, bone, tendon, dentin, cornea and fibrocartilage (van der Rest & Garrone 1991). The presence of type I collagen in developing cartilaginous rudiments is well established. However, in mature adult articular cartilage there are conflicting opinions. In bovine (Wu & Eyre 1995) and human mature articular cartilage, collagen type I cannot be isolated. In a porcine model, however, type I collagen was found to be located at the articular surface and in the pericellular environment of hypertrophic chondrocytes in the deep zones (Wardale & Duance 1993). mRNA for type I collagen has been isolated from human adult articular cartilage but there is no evidence that this is translated into collagen type I. The general impression following immunolocalisation studies is that collagen type I as in the case of type X is synthesised by hypertrophic chondrocytes both intracellularly and pericellularly (Morrison et al. 1996b) but not usually found in normal mature healthy articular cartilage. Although chondrocytes in normal articular cartilage do not produce collagen type I, in monolayer cultures, chondrocytes are known to dedifferentiate to fibroblast like cells and produce collagen type I (Shakibaei & De Souza 1997).

Collagen type III is a homotrimer with three identical α-chains. It is found mainly in soft connective tissues such as blood vessels, skin, muscle, and placenta. Although, thought not to be a component of normal healthy articular cartilage, there is evidence that it exists in developing and diseased articular cartilage (Morrison et al. 1996c).

Proteoglycans

Articular cartilage contains two classes of proteoglycans: Large aggregating proteoglycan monomers or aggrecans, and small proteoglycans including decorin, biglycan, and fibromodulin (Buckwalter & Mankin 1998d). Proteoglycans are complex macromolecules that, by definition, consist of a protein core to which are linked extended unbranched glycosaminoglycan chains. Proteoglycans were formerly called protein-polysaccharides or mucopolysaccharides and this term is still used to describe some inherited storage disorders.

The glycosaminoglycan (GAG) side chains are composed of repeating disaccharide units. The molecules are referred to as GAG because one of the two sugar residues is always an amino sugar, N-acetylglucosamine or N-acetyl galactosamine. The second sugar may be one of the uronic acid residues D-glucuronic acid or L-iduronic acid or the hexose sugar, D-galactose. The glycosaminoglycans present in living tissues have many sulphate and carboxylate groups and are therefore strongly negatively charged. The GAGs provide cartilage with a high concentration of fixed negative charge. The sulphated GAGs include chondroitin sulphate, keratan sulphate, dermatan sulphate, heparan sulphate and heparin (not present in articular cartilage).

Eighty to 90% of all proteoglycans in cartilage are of the large, aggregating type, termed aggrecan. It consists of a large, extended protein core to which are attached up to 100 chondroitin sulphate and 50 keratan sulphate glycosaminoglycan chains. The protein core of aggrecan is large with a molecular weight 2.25 KD and complex, and has several distinct globular and extended domains. One extended domain contains the majority of the keratan sulphate chains, and is adjacent to the longest extended region, which has the chondroitin sulphate chains attached with some keratan sulphate chains interspersed. Finally, some small oligosaccharides are also attached along the protein core.

At the N-terminal end of the protein core, one of the globular domains (G1) has the specific function of binding to hyaluronate. The function of the other globular domains of aggrecan is unknown. A separate, smaller molecule called the link protein binds to both the G1 domain of aggrecan and the hyaluronate, stabilising the bond and, thus, forming an aggrecan-hyaluronate-link protein complex. The hyaluronate therefore acts as a backbone from which the aggregates span out. The noncovalent interactions of this complex are so strong that without proteolytic degradation this binding can be regarded as almost irreversible.

Because each hyaluronate chain, which is a non-sulphated GAG, is long and unbranched, many aggrecan molecules can bind to a single chain of hyaluronate to form a large proteoglycan aggregate. Aggregate sizes can vary with age and disease state, but each aggregate can contain up to 200 aggrecan molecules. In this way, large proteoglycans are thought to become immobilised within the collagenous network of the cartilage (Mankin & Thrasher 1975).

Proteoglycan monomers, link proteins and hyaluronan are secreted independantly from the chondrocyte and are assembled as aggregates within the extracellular matrix. As part of the normal matrix turnover, most of the proteoglycan core protein is enzymatically cleaved releasing large proteoglycan fragments. The mechanisms of synthesis and digestion are co-ordinated such that the total amount of proteoglycan remains constant in adult human articular cartilage (Hardingham & Bayliss 1990).

The structure and composition of proteoglycans varies with age (Mort, Poole, & Roughley 1983), site (Bjelle 1975) and depth of cartilage (Buckwalter, Roughley, & Rosenberg 1994) (Jones, Larsson, & Lemperg 1977). The proportion of keratan sulphate increases and chondroitin sulphate decreases with increasing depth which implies a reduction in the relative size of the proteoglycan monomer and therefore the proteoglycan aggregate (Jones & Lemperg 1978). This may explain the reason for the higher water content of cartilage in the superficial zone

Non-collagenous proteins

The non-collagenous proteins consist primarily of protein and have a few attached monosaccharides and oligosaccharides , but are not as well understood as the collagens and proteoglycans. They include link protein, chondronectin and anchorin CII. The latter is a collagen-binding chondrocyte surface protein which may help to anchor chondrocytes to the collagen fibrils of the matrix. Chondronectin also mediates adhesion of collagen fibrils to chondrocytes and may help to stabilise the chondrocyte phenotype. Cartilage oligomeric protein, an acidic protein, is concentrated primarily within the territorial matrix and has the capacity to bind to chondrocytes (Hedbom et al. 1992). It may function as a marker in regulation of cartilage turnover and has been implicated in the progression of cartilage degeneration in osteoarthrosis (Lohmander, Saxne, & Heinegard 1994). Fibronectin and tenascin, non-collagenous matrix proteins found in a variety of tissues have also been identified within cartilage (Hayashi, Abe, & Jasin 1996). Although their function is unknown, they may have roles in matrix organisation, cell-matrix interactions, and the response of the tissue in inflammatory arthritis and osteoarthrosis.

Tissue Fluid

Water is the main constituent by weight and makes up to 80% of the wet weight of cartilage. Some of it can move freely within the tissue and the remainder is closely associated with large aggregating proteoglycans that help to maintain the fluid within the matrix and the concentrations of electrolytes in the fluid (Maroudas & Schneiderman 1987). Proteoglycans in matrix are only partly hydrated and have a constant tendency to expand. However, the swelling pressure of the highly negatively charged and densely packed proteoglycan aggregates is opposed by the tensile stresses established within the of type II collagen fibrils in which the proteoglycans are entrapped (van der Rest & Mayne 1988).

Collagen itself also is hydrophilic but less so than the proteoglycans. The water content of cartilage is non-homogeneously distributed, decreasing in concentration from approximately 80% at the surface to 65% in the deep zone. Most of the water may be moved through the tissue or squeezed out from the tissue by the application of either a

pressure gradient across the tissue or by compressing the solid matrix. The frictional resistance against this flow through the molecular size pores of the extracellular matrix (ECM) is very high, and thus the permeability of the tissue is very low. This frictional resistance and the pressurisation of the water within the ECM are the two basic mechanisms from which articular cartilage derives its ability to support very high joint loads (Mow et al. 1980).

2.2 Articular Cartilage Damage and Response

Although injuries to joints are common in mammalian species including humans, it has been known for a long time that the healing capacity of articular cartilage is poor. Hunter as early as 1743 stated that 'from hippocrates to the present age it is universally allowed that ulcerated cartilage is a troublesome thing and once destroyed, it is not repaired'. In 1853 James Paget reported that 'there are no instances in which a lost portion of cartilage has been restored, or a wounded portion repaired with new and well formed cartilage'. Although mechanical trauma is an obvious form of injury to articular cartilage, more subtle forms of injury include the following;

Joint infection or inflammation
Prolonged joint immobilization
Abnormal joint loading
Drugs (ie anti-inflammatory agents)
Degenerative Disease processes
Ligament and meniscal injuries predisposing to articular cartilage damage.

2.2.1 Prolonged Joint Immobilisation

Maintenance of normal cartilage structure, composition and function requires a minimal of joint loading and motion. In dogs, depletion of proteoglycans was observed after 11 weeks of immobilisation and was not completely restored in certain surface sites after 50 weeks of remobilisation. These results indicate permanent alteration of matrix metabolism may result after even a temporary modification of loading pattern in immature joints (Jortikka et al. 1997). Increased joint loading and motion, up to a certain level, may increase cartilage matrix degradation. In dogs, increased loading of a

limb, resulting from cast immobilization of the opposite limb or moderate running exercise (4km/day, increased cartilage glycosaminoglycan concentration and thickness. Yet strenuous running (20 km/day) reduced cartilage thickness and GAG concentration in normal joints, suggesting that loading and motion above a certain level may adversely affect articular cartilage (Kiviranta 1987).

2.2.2 Penetrating Trauma

Penetrating trauma to articular cartilage is rare, however it may occur during twisting or direct violence to synovial joints especially during vigorous activity. The resulting shear forces applied to an articular surface can rupture the cartilage matrix producing chondral fissures, flaps or fractures. If confined to the substance of the cartilage (not violating the junction of the calcified zone and the underlying osseous end-plate) there is classically an absence of the typical inflammatory response of cell migration to the site of injury as occurs in more vascular tissue (Mankin 1982). The injury creates a wedge shaped matrix defect resulting in chondrocyte death of about 50-100ηm region around the margins of injury. This phase of necrosis in which ghost cells are observed in the lacunae of chondrocytes adjacent to the margins of the wound follows immediately after injury. At about 24 hours there is an intense burst of mitotic activity in chondrocytes adjacent to the defect. This activity is associated with markedly enhanced rates of cellular synthesis of matrix components, as measured by the incorporation of $^{35}SO_4$ (an indicator of glycosaminoglycan synthesis) and 3H-glycine (an indicator of protein synthesis) (Mankin 1982). Histologically, nucleolar hypertrophy, increased quantities of rough surfaced endoplasmic reticulum and increased numbers of golgi bodies complexes may be noted (Fuller & Ghadially 1972), This increased activity around the defect accelerates through the first few days but is short lived. By two weeks the increased activity diminishes to normal cartilage levels. After this initial period, the lesions remain stable and do not usually progress to osteoarthritis. Although the lesions do not progress to osteoarthritis, electron microsopic observations after 2 years post injury have revealed that there is no healing of the lesions and they remain histologically similar to soon after injury.

Deeper defects which penetrate into subchondral bone result in a different healing process as inflammatory cells from disrupted interosseous blood vessels are able to migrate to the injury site and the repair response is therefore much more characteristic

of that seen in other vascularised tissues. The defect is filled with a blood clot and mesenchymal osteochondral precursor cells and white blood cells proliferate and undergo differentiation into primitive fibroblasts (Mankin 1982). Gradually the defect becomes more cellular and less vascular (Campbell 1962). Fibrocartilaginous or hyaline cartilage like tissue is formed. This form of repair tissue although resembling hyaline cartilage differs both in its biochemical and mechanical properties. It is composed of much greater quantities of collagen type I which is unlike hyaline cartilage were collagen type II is the primary constituent (Furukawa et al. 1980). By twelve months after injury the surface layer of cells lack the typical tangential arrangement seen in normal cartilage. With time the surface may become fibrillated and the subjacent matrix densly collagenous. Although mechanically the repair tissue is inferior to normal articular hyaline cartilage (Coletti, Jr., Akeson, & Woo 1972), the abnormal repaired cartilage functions reasonably well providing the initial defect was less than nine millimetres in diameter. Larger lesions tend to heal incompletely (Convery, Akeson, & Keown 1972) and would probably function less well.

2.2.3 Blunt Trauma

Excessive joint loading and direct blunt trauma subject articular cartilage to intense compression which does not occur in normal loading. In normal loading, the cartilage undergoes surface compaction with the lubricating fluid being exuded throughout this compacted region near the surface. Within the cartilage, fluid redistribution occurs, to relieve the stress. If the compaction occurs too rapidly for compensation to occur, high stresses are produced which may result in damage to the collagen-proteoglycan complex. Disrupting normal articular cartilage with a single impact requires substantial force. A comprehensive study of human articular cartilage subjected to blunt trauma by a drop-tower technique showed that articular cartilage could withstand impact loads of up to 25 N/mm^2 (25 MPa) without apparent damage (Repo & Finlay 1977). A further study concluded that impact loads of magnitudes that do not fracture bone may be sufficient by causing stresses greater than 25 Mpa in some regions to disrupt cartilage (Haut 1989).

Repetitive impact loads may cause splitting of articular cartilage matrix and initiate progressive cartilage degeneration. Chondrocyte clusters, fibrillation of the matrix,

thickening of the subchondral bone, penetration of the subchondral capillaries into the calcified zone of articular cartilage are all evidence of cartilage damage (Dekel & Weissman 1978). The extent of cartilage damage increases with longer periods of repetitive overloading and may continue after the excessive loading has stopped. This implies that damage to cartilage matrix may occur without significant visible changes. Loss of proteoglycans or alteration in their organization (especially decrease in proteoglycan aggregation) caused by increased degradation or decreased synthesis of proteoglycan molecules may lead to decreased cartilage stiffness and increased hydraulic permeability. These alterations cause greater loading of the remaining macromolecular framework, including the collagen fibrils, thereby increasing the vulnerability of the tissue to further damage from impact loading. Chondrocytes themselves may be injured as a result of the loss of collagen fibril-proteoglycan relationships (Buckwalter & Mankin 1998a).

2.3 Articular Cartilage Response

Partial-thickness defects in articular cartilage do not heal spontaneously. Injuries of the articular cartilage that do not penetrate the subchondral bone do not heal and usually progress to the degeneration of the articular surface. Injuries that penetrate the subchondral bone undergo repair through the formation of fibrocartilage.

Although fibrocartilage fills and covers the defect, this is abnormal tissue from the biomechanical standpoint. Fibrocartilage is made to resist tension forces, while the hyaline cartilage is made to resist compression forces, to enable smooth articulation, and to withstand long-term variable cyclic load and shearing forces. Focal articular cartilage defects, often found in young adults, have been increasingly recognized as a cause of pain and functional problems.

There is greater clinical evidence that full thickness articular cartilage defects continue to progress and deteriorate, although at a slow rate. Early diagnosis and treatment of these patients is recommended prior to the development of more advanced osteoarthritis.

2.4 Articular Cartilage Repair

Functional articular cartilage is critical to proper joint function. Unfortunately, as discussed earlier, after injury it has a poor capacity to repair itself and in the long term this may lead to osteoarthritis. This is particularly true for a large weight bearing joint such as the knee. The mechanisms that control articular cartilage repair are at present poorly understood, however certain factors are known to influence the repair response.

Continuous passive motion has been shown to stimulate the healing of full thickness articular cartilage defects but immobilisation and intermittent active motion do not appear to have the same beneficial effect (Salter et al. 1980). The mechanism by which this occurs may be related to the availability of increased amounts of synovial fluid in the joint during passive motion. Most workers consider the synovial fluid to be the chief source of nutrition in articular cartilage (Zahir & Freeman 1972) and it has been shown that physiological exercise increases the volume of synovial (Ekholm & Norback 1951). If it is assumed that the converse is true, there will be a decrease in the volume of synovial fluid in an immobilised joint with the diminished possibility of nutrition as a result. Immobilisation also allows formation of intra-articular adhesions which may explain the poorer results in joints that are only intermittently exercised (Salter, Simmonds, Malcolm, Rumble, MacMichael, & Clements 1980).

Pluripotential mesenchymal cells can differentiate along different lines depending on both physical factors and oxygen tension. Compaction and a high oxygen tension cause the primitive cells to form bone, compaction and low oxygen tension cause them to produce cartilage whereas tensile force and a high oxygen tension influence them to produce fibrous tissue (Basset 1962)

Growth factors have influence on the synthesis of cartilage matrix components and chondrocyte activity. Insulin like growth factor (IGF-1) has a profound effect on proteoglycan synthesis (sulphate incorporation) with a lesser effect on cell proliferation (thymidine incorporation) in vitro (Osborn, Trippel, & Mankin 1989). Basic fibroblast growth factor (bFGF) stimulates proliferation of articular chondrocytes in vitro and has been shown to accelerate repair of superficial articular cartilage defects in vivo (Cuevas, Burgos, & Baird 1988) Transforming growth factor beta (TGF-b) is implicated in

regulating both proteoglycan synthesis and proliferation of articular chondrocytes (Brenner et al. 1993).

The natural repair processes result in a repair cartilage which is deficient in its articular functional properties. Due to the incorrect material properties and composition it becomes fibrillated and eventually undergoes degeneration (Furukawa, Eyre, Koide, & Glimcher 1980;Mitchell & Shepard 1976). Despite these long established facts one may question the reasons why investigators continue to search for methods of inducing the restoration of articular surfaces and surgeons continue to perform operations intended to stimulate repair of damaged.

Treatments such as arthrodesis and total joint arthroplasty are frequently not a satisfactory solution for young patients because the procedures have a limited life span (they loosen during skeletal development) and they have a restricted tolerance for heavy loading and vigorous activity. For young patients the option of using a procedure which stimulates cartilage repair, even if it does not restore a normal functional articular surface, is a feasible one. Even if the repair tissue only lasts a few years it can potentially improve their quality of life, allowing them to remain physically active and delaying the option of total joint arthroplasty until they reach skeletal maturity (Buckwalter & Mow 1990). In some instances the use of surgical procedures can stimulate the formation of new joint surfaces which compare favourably with artificial joints and arthrodesis (Dell & Muniz 1987). For these reasons and other reasons, investigators continue to search for a means of stimulating cartilage repair that will create an optimal biological and mechanical repair environment to produce a functional alternative that will replace the injured cartilage.

Many approaches have been used with varying success rates, the methods have been diverse with frequently disparate grading systems.. To demonstrate the superiority of a certain technique, it must be proven to achieve better long-term clinical results than the natural history, and current methods of treatment. Since long-term results for these procedures may mean as much as 20 to 30 years of follow up, an alternative standard may be to ascertain how closely the regenerative tissue resembles articular cartilage structurally, biochemically and mechanically (Newman 1998). The more closely the native structure of articular cartilage is replicated, the more likely it is that the tissue will result in normal, symptom-free joint function and will not deteriorate prematurely

over time. Some of the methods being employed to achieve this goal will be described in the following sections.

2.4.1 Non Biological Repair : Surgical treatment

2.4.1.1 Changing Loads on Articular Cartilage

Although, not a method of cartilage repair in itself, osteotomies have been used frequently in the knee and hip as a means to decrease symptoms of osteoarthritis. The realignment of the joint reaction force towards a remaining healthy surface has been beneficial for upto 12 years in some patients (Weisl 1980). Apart from the shifting of joint contact to a more healthy cartilage covered articular surface there is some evidence that the change in loading conditions may actually stimulate the diseased surface to initiate repair and reconstititution of a thicker articular cartilage (Itoman et al. 1992) (Buckwalter & Mankin 1998a).

2.4.1.2 Shaving of fibrillated or damaged cartilage

Debridement of damaged articular cartilage may relieve symptoms in two ways. Some cartilage, either by formation of unstable flaps or from fibrillation or other distortions of anatomy, may create mechanical problems, such as locking, catching, or crepitus. In addition cartilage debris can induce a synovitis and joint effusion (Evans et al. 1984). Debridemant can decrease these mechanical and inflammatory symptoms. There is no evidence that shaving of superficial cartilage stimulates repair. This is not surprising when one considers the repair processesfor superficial lesions discussed earlier. There is experimental evidence to suggest that there is increased fibrillation and necrosis in adjacent hyaline cartilage (Kim, Moran, & Salter 1991). The relief therefore is only temporary.

2.4.1.3 Perforation and Abrasion of Subchondral bone

Penetration of cartilage to subchondral bone exposes growth factors and inflammatory mediators to the defect which in turn stimulates the synthesis of repair matrix. A variety of techniques have been employed including multiple drill holes (Insall 1974) or microfractures, spongialization (Ficat et al. 1979) and abrasion (Bert 1993) of a few

millimetres of the subchondral cortex. The results of these procedures have been variable. The use of drill holes promotes the healing responses and recovery of an apparently normal articular surface in the initial phase; however, in the long term the holes become more collagenous and deteriorate within one year (Coletti, Jr., Akeson, & Woo 1972). In one study multiple perforations created in the femora of rabbit knees resulted in complete resurfacing of the joints at one year, but the material resembled dense collagenous tissue (Mitchell & Shepard 1976). Microfracture is a marrow stimulation technique in which the subchondral bone is exposed, gently abraded, but left intact, while adjacent cartilage is debrided to healthy cartilage. The subchondral bone is then broached using small picks so that it communicates with the marrow and clot formation is stimulated. A study by Akeson et al (Akeson et al. 1969) examined the tissue formed after removal of all cartilage and subchondral bone from dog femoral heads and a vitallium cup interspaced between the cancellous bone of the femoral head, a nonuniform articular surface resulted that showed variability between animals. There were differences in the response in different regions of the femoral head and the cartilage of the acetabulum, and in the long term there were abnormal levels of proteoglycan and collagen in the repair tissue. In spite of these problems, some successes have been achieved in humans using these and other procedures in promoting cartilage healing.

2.4.2 Biological repair : Treatment with transplanted tissue

Due to the limitations of hyaline cartilage healing and the variable results of the treatment modalities previously described, there is much interest in the transplantation of cartilage tissue and isolated chondrocytes. If intact tissue (chondrocytes with their surrounding matrix is used, current practice is to transplant an osteochondral unit (with underlying bone) to capitalize on bone-to-bone healing, since the cartilaginous cap itself will not bond to recipient bone or cartilage (Garrett 1998). Although research with various biological adhesives such as transgluataminases (Jurgensen et al. 1997) and fibrin sealants (Brittberg et al. 1997) suggests that effective cartilage-cartilage bonding may be feasible in the future, this approach is not yet clinically practicable.

2.4.2.1 Osteochondral Autograft/Allograft

The treatment of disabling cartilage lesions whether limited to small areas of the articular surface or much larger areas has been by total joint replacement. A biological alternative for the replacement of limited injured or portions of the articular surface has been the subject of considearble experimentation since the beginning of the twentieth century. In 1908, Lexer reported the first series of fresh osteochondral transplants into human joints, and he stated that the function of the joint was good in each patient after incorporation of the allograft (Meyers, Akeson, & Convery 1989).

An area of significant interest regarding allografts is the immunogenicity of the transplanted material. Articular cartilage is considered to be an immunologically priveleged tissue because its avascularity isolates it from the immune system, and macromolecules within the matrix are only weakly immunogenic (Convery, Meyers, & Akeson 1991). However, when transplanting osteochondral grafts, cartilage tissue must be transplanted with attached subchondral bone. Fresh allograft bone possesses immunogenic cells resulting in lymphocytic infiltration and some components of immunologic rejection. One way of inhibiting this response is to freeze the osteochondral unit. Freezing reduces the immunogenicity, but also decreases the viability of the transplanted chondrocytes (Schachar et al. 1992). Desjardins et al (Desjardins, Hurtig, & Palmer 1991)studied fresh and frozen autogenous grafts in horses. They found that fresh grafts had a higher percentage of living cells, as well as higher proteoglycan content, than frozen grafts. The fate of the cartilage was influenced by the congruence and fit of the grafts, with deeply recessed grafts faring poorly, being replaced by fibrous tissue.

Autogenous osteochondral grafts have some advantages over allograft, including no threat of disease transmission, high survival rate of the grafted chondrocytes, and reliability of bone union. Disadvantages include limited supply of autogenous tissue, morbidity or adverse effect on joint function or graft harvest, fixed age of donor (ie not being able to use tissue from younger donors with correspondingly enhanced ability to generate repair tissue), and the difficulty of matching topology of graft to recipient site (Newman 1998). Although current tissue banking techniques reduce the risk of disease transmission to extremely low levels, it is unlikely to be ever completely eliminated.

Clinically, Outerbridge first described the use of a lateral patellar autograft for the repair of large osteochondral defects in the knee. Femoral condyle defects were repaired with autologous grafts harvested from the lateral facet of the patella and press fit into place (Outerbridge, Outerbridge, & Outerbridge 1995). In the ten patients who underwent surgery and followed up for 61/2 years all had good relief of symptoms with minimal donor site morbidity. Success rates of greater than 80% measured clinically have been reported in some studies. Unloading osteotomies are often needed to prevent collapse and failure of grafts. Meticulous attention to matching size and inlay fixation are critical to success.

2.4.2.2 Perichondrial Grafts

In an effort to use autogenous tissue without the morbidity associated with the use of articular cartilage, investigators have turned to other tissues capable of chondrogenesis to repair articular cartilage defects. The chondrogenic potential of articular cartilage perichondrium was documented by Tizzoni in 1878. In the early 1900's, Haas described the formation of cartilage from the perichondrium of the rib (Haas 1914). However, only in the last two decades have clinical applications been considered for this progenitor tissue. In mammals, the perichondrium forms the outer layer of rib and ear cartilage. It develops from the embryonic mesoderm and consists of several layers including an outer fibrous layer containing collagen fibres and an inner layer of oval mesenchymal cells capable of chondrogenesis (Bulstra et al. 1990). The potential of human perichondrium to form hyaline cartilage in vitro was clearly demonstrated by Bulstra et al. Amiel et al (1985) showed that in immature adult animals free autogenous perichondrial grafts of the rib were able to form hyaline cartilage when placed in an artificial cartilage defect in a joint. On gross morphology at twelve weeks the neocartilage was confluent with the surrounding cartilage. The only difference was the slightly lighter colour of the neocartilage which demarcated it from the surrounding pinkish articular cartilage. Biochemically it had high concentrations of type II collagen and glycosaminoglycans. Although the grafts were stable up to one year after implantation, they still contained significantly more type I collagen than is present in normal articular cartilage (Woo et al 1978) The differentiation of perichondrium to form hyaline cartilage is dependent on extrinsic influences emanating from its environment. The presence of motion, low oxygen tension, and absence of vascularity of the synovial

environment could favour and maximise its development. Further technical developments may make perichondrial grafting an attractive clinical tool in the future. Graft size is limited by rib size, and several ribs often must be used to obtain sufficient perichondrium to graft multiple or large defects (Minas & Nehrer 1997). This is a major limitation in using perichondrium.

2.4.2.3 Periosteal Grafts

Periosteum is a resiliant, vascular, fibrous tissue which surrounds bone. Embryonically it is derived from the perichondrium (Rubak, Poussa, & Ritsila 1982). The mesenchymal cells in the lower layer termed the cambium have the potential for chondrogenesis and osteogenesis (Ham 1930). As with perichondrium, the biological environment determines the phenotypic expression of the graft (Itay, Abramovici, & Nevo 1987a). Circulation of the recipient bed and the level of oxygen tension are important; high oxygen tension favours bone production, and low oxygen tension favours cartilage production. O'Driscoll et al (1984) (O'Driscoll & Salter 1984)showed that autologous perichondrium obtained from proximal tibia of adolescent and adult rabbits under the influence of continuos passive motion could repair full thickness defects of the patellar groove and demonstrated the durability of the regenerated surface after 1 year. Chondrocyte cell clusters were found at about 4 weeks after repair with significantly fewer clusters identified at 1 year. However, Moran et al noted abnormalities in the arrangement and distribution of type II collagen and found some evidence of chondrocyte degeneration in the neocartilage (Moran, Kim, & Salter 1992).

2.4.2.4 Mesenchymal Stem Cells

As cells differentiate, their phenotypic expression becomes more specialised to their microenvironment, and they become less able to adapt to other circumstances (Newman 1998). Thus, in tissue from a mature animal, the cells are relatively 'frozen' into a single manifestation of their genetic potential. This creates a problem in the use of chondrocyte transplantation. Chondrocytes from the superficial zone create a very different matrix than cells from the deep zone, and vertebral disk chondrocytes have a very different genetic expression from knee chondrocytes (Caplan, Goto, & Wakitani 1992). Some studies have shown the capacity of implanted chondrocytes to develop according to their topographic location in the host, apparently because of the control

that the microenvironment has on the cellular differentiation and phenotypic expression (Itay, Abramovici, & Nevo 1987b). In theory, the use of undifferentiated stem cells would allow greater adaptation to the microenvironment than mature cells, resulting in a structure that more closely resembles the native tissue.

Osteochondral progenitor cells have been isolated from periosteum and bone marrow, and then mitotically expanded to generate large numbers of autologous mesenchymal stem cells (Caplan, Goto, & Wakitani 1992). There are two theoretical advantages to the use of these stem cells. First, the target of the bioactive molecules released by injured bone and blood vessels is the mesenchymal stem cell, not the mature, differentiated chondrocyte. Second, because these progenitor stem cells are less differentiated than mature chondrocytes, they are capable of a broader range of chondrogenic expression (Wakitani et al. 1989) and may manifest a superior ability to replicate the normal microarchitecture and biochemistry of articular cartilage in the site to which they are transplanted.

Wakitani et al (1994) successfully transplanted mesenchymal stem cells into rabbit knee full-thickness defects with results as good as if not better than the repair instituted by allogenic articular chondrocytes (Wakitani et al. 1994). Hyaline-like cartilage with good mechanical properties was present in the defects at 6 months.

2.4.2.5 Chondrocyte Transfer

There has been a fairly long history of interest in the transplantation of isolated chondrocytes to achieve the healing of cartilage lesions. Techniques for the isolation and culture of chondrocytes were developed in the early 1960's. Chesterman and Smith (1968) reported the results of implanted isolated immature rabbit chondrocytes into full thickness defects of rabbit humeral articular cartilage (Chesterman & Smith 1968). Examination of grafts after 12 weeks revealed the formation of mainly fibrocartilaginous repair tissue. Bentley and Greer in 1971 demonstrated a better chondrocyte survival rate using isolated epiphyseal chondrocytes rather than articular cartilage chondrocytes when they allografted cells into the upper tibial articular surfaces of adult rabbits (Bentley & Greer, III 1971). Aston and Bentley in 1982 cultured articular and epiphyseal chondrocytes for up to 6 weeks. They produced 30 times the number of original cells and found that these cells produced a matrix similar to hyaline cartilage that was positive for type II collagen (Aston & Bentley 1986). In subsequent studies they used these cultured cells as allograft transplants into rabbit knees, and they

found 64% successful incorporation at 1 year. There was no evidence of rejection, and failures were all due to mechanical loosening of the graft at an early stage. Grande et al in 1989, used autologous chondrocytes and showed approximately 82% coverage of patella articular cartilage defects as compared to 18% for control defects at one year in rabbits (Grande et al. 1989).

Autologous chondrocytes produce better early healing of defects than allografts due possibly to the more rapid formation of subchondral bone. Allografted specimens result in a delayed subchondral bone formation response which, although investigators have not been able to confirm, may have an immunologic basis. This could have clinical implications on time of postoperative weight bearing and therefore in the clinical setting, autologous chondrocyte transplantation would seem to have an advantage.

Histological analysis of defects treated with autologous chondrocytes reveals 3 stages of healing (Itay, Abramovici, & Nevo 1987c). The *proliferative stage* takes place during the first 4 weeks and is characterised by a rapid increase in the number of chondrocytes and a small increase in glycosaminoglycan production. The *Maturation stage* follows and cartilage is formed. The cells differentiate, presumably based on their location within the defect and as a response to local nutritional and mechanical factors. As rates of cellular proliferation decreases, the rate of matrix synthesis correspondingly increase. The final stage occurs between 2 and 6 months after transplantation and is termed the *transformation stage*. In the subchondral region, vascular elements proliferate and penetrate the subchondral portion of the implant. Chondrocytes in this region undergo degenerative changes and are replaced by primary osteons, and the subchondral bone plate is reconstituted. Although the exact nature of the events may vary depending on certain factors (such as depth of defect, use of synthetic or biological carrier systems, and addition of growth factors), this sequence is consistent with the general understanding of chondrogenesis and with observations on other experimental studies of chondrocyte transplantation.

In 1994, news media all over the world presented a new cure for knee cartilage lesions that was suggested to eliminate the need for 500,000 artificial joint replacements per year. This was based on the article by Brittberg et al (1994) in the New England Journal of Medicine (Brittberg et al. 1994). The proposed technique was later made commercially available by Genzyme Tissue Repair (Boston, Mass., USA). Twenty three

patients with a mean age of 27 years having cartilage defects ranging in size from 1.6 to 6.5 cm^2 on the load bearing surface of the the femoral condyle were treated with the new procedure. Autologous chondrocytes, isolated from cartilage fragments obtained from minor weightbearing regions of the femoral condyle in the damaged knee, were cultured and expanded in vitro using monolayer techniques. Two to three weeks later the cells were reimplanted into the patients knee. Damaged cartilage was excised down to subchondral bone without violating the bone. A periosteal cover approximately the same size as the defect harvested from the upper medial tibia and sutured to the edges of the defect. The cultured cells were injected under the periosteal cover. The patients were allowed range of motion of the knee without weightbearing initially, with weightbearing gradually introduced over 2 to 3 months. Two years after transplantation 87% of patients had good to excellent results ie restoration of uninhibited joint motion and reduced pain.transplanted into the defects. On biopsy 73% had hyaline-like cartilage; the remainder demonstrated a fibrocartilage appearance. Immunohistochemical testing for type II collagen was positive but, ratios or amounts of Type I and Type II collagen were not reported. Other criticisms of this study are that it was not a randomised trial, there was not a comprehensive histological examination of the lesions and outcome analyses were subjective (Newman 1998).

2.4.2.6 Three dimensional synthetic and biological matrices

Tissue engineering is a concept which has emerged recently as a new form of therapy in which synthetic materials seeded with appropriate cell populations are grown in vitro and subsequently implanted into patients. Polymers are being used to fabricate the so called 'designer tissues' (skin, liver, nerve, blood vessel, and intestinal lumen) in the laboratory. Using specific cells incorporated into a polymer matrix, the three dimensional architecture of the cultured tissue is recreated which allows a more natural environment for cellular differentiation and expression of phenotype.

It is well established that cells constantly interact with each other and with their surrounding local microenvironment, and this communication serves to integrate and coordinate the various gene expression patterns that are crucial for tissue function and homeostasis. Two major signal pathways participate in the cooperative cell communication process. The first pathway involves the secretion of soluble growth factors and control factors into the microenvironment by the surrounding cells or, in the

case of hormones, by cells in distant tissue microenvironments. This signaling process has been recognised for many years, and a number of individual signaling cascades can now be described in intricate biochemical detail. The second major pathway for cooperative signalling is less well understood. It involves the constant feedback signals generated by the direct contact of cells with the components of their extracellular matrix (ECM). Originally thought to supply only structural support for the cells of a tissue, the ECM is now known to be critical for regulating cell morphology, proliferation, and differentiation, and is capable of responding to various endogenous and exogenous stimuli. The ECM transmits important positional and mechanical signals to the cytoskeleton, these signals are transmitted to the nuclear matrix proteins, which act to control the expression of various genes by the cells of a tissue. This collection of cells, extracellular matrix, cytokines, and growth factors comprise a microenvironment that is the basis of normal tissue structure and function.

There would be numerous advantages inherent in such an approach as applied to articular cartilage. The need for donor tissue would be decreased , through in vitro amplification of autogenous or allogenic chondrocytes.. The synthetic scaffolds, by providing an initial framework, could temporarily stabilize the chondrocytes in the defect and direct their spatial distribution, before their synthesis of collagen and proteogycan. Given a material of suitable mechanical properties (shape-retaining but malleable), arthroscopic implantation may be feasible. They can be employed as carriers for various growth factors and other bioactive molecules to enhance the environment for cartilage healing. The repair of larger surface defects (in theory, entire condyles) could be addressed by the manufacture of chondrocyte-polymer composites with specific three-dimensional shapes. The initial mechanical properties, as well as the rate of biodegradation, could be manipulated to support the healing process.

The initial work on in this area centered on using matrices constructed from natural extracellular matrix (ECM) proteins such as collagen (Yannas 1988)for skin cells and has been applied to other tissues such as blood vessels. The successful application of matrices made purely from ECM proteins to tissues such as cartilage may be limited by their poor mechanical properties. Fibrinogen based materials, collagen gels, carbon fiber pads, and polylactic and polyglycolic acid meshes have been used as matrix materials. Hyaline cartilage matrix was produced, differentiation of cells and inhibition of granulation tissue formation was demonstrated by (Itay, Abramovici, & Nevo 1987d)

when chick embryonal chondrocytes were impanted as allografts embedded in a biological resorbable matrix composed of fibrinogen, thrombin and an antiprotease substance. Polylactic acid (PLA) cores were used by to repair articular defects in rabbit knees (Billings, Jr. et al. 1990). Consistent repair of the articular defects with hyaline cartilage , containing 82% Type II collagen. A further study by Chu et al reported successful implantation of PLA constructs into rabbit knees with evidence of seamless integration with the host cartilage, however, high levels of collagen type I was detected in the repair tissue (Chu, Monosov, & Amiel 1995).

In 1993 Freed et al reported on the use of synthetic, biodegradable polymer scaffolds made of fibrous Polyglycolic Acid (PGA) and porous PLA (Freed et al. 1993). They were seeded with either bovine or human chondrocytes, which then grew in culture. The chondrocytes produced cartilage matrix filling the void spaces in the polymer scaffolds, as they were simulataneously biodegrading. Freed et al also used allograft chondrocyte - PGA composites to repair full thickness defects in adult rabbit knees. The 6-month repair tissue was superior to that seen with PGA alone with respect to smoothness, columnar orientation of chondrocytes, reconstitution of the subchondral plate, and bonding of the repair tissue to the underlying bone and the host tissues from the sides. They observed that the chondrocyte-PGA composite, unlike other constructions, was firm enough to be cut and shaped, while at the same time it was flexible enough to be potentially delivered through an arthroscopic cannula.

Several criteria define the ideal material for a cell transplantation matrix (Cima et al. 1991). The material should be biocompatible, in the sense that it does not provoke a connective tissue response which will impair the function of the new tissue; resorbable to leave a completely natural tissue replacement; easily and reproducibly processable into a variety of shapes and structures which retain their shape once implanted; and finally, the surface of the material should interact with transplanted cells in a way which allows the retention of differentiated cell function and which promotes cell growth function if such growth is required. In consideration of the desired properties above, materials which may have previously been used for cell transplantation have inherent limitations which may exclude them from becoming clinically useful as substrates for regenerating permanent tissue. Non-resorbable materials carry a permanent risk of infection and undesired connective tissue reaction. Resorbability has been shown to be

an important criterion for chondrocyte transplantation in matrices (Vacanti et al. 1991) and the resorption rate, if too fast, may not allow adequate formation of cartilage.

The majority of clinical studies on cartilage repair can be criticised on lack of controls, lack of randomisation, poor outcome analysis, and lack of biochemical and biomechanical data. However, it seems that progress is being made. The centuries-old supposition that articular cartilage is incapable of healing has been severely shaken and seems to fall. Advances are being made on a number of fronts, and perhaps some combination of chondrocyte transplantation in three dimensional matrices and pharmocologic intervention will combine to yield a satisfactory solution.

2.5 Three dimensional scaffold cultures

2.5.1 Background

As described earlier, cells constantly interact with each other and with their surrounding local microenvironment, and this communication serves to integrate and coordinate the various gene expression patterns that are crucial for tissue function and homeostasis. Soluble factors released into the extracellular matrix are important for not only the signalling processes between cells but also it is now recognised that the direct contact of cells with matrix components is essential in many tissues (Sommarin, Larsson, & Heinegard 1989).

Many aspects of cell behaviour that appear incidental or are unrecognised when cells are studied as individual entities become of paramount importance in the organised three dimensional structure of tissue. Anchorage dependant fibroblasts, for example, when placed in suspension culture systems lead to an extensive shut down of all major macromolecular processes. The loss of surface contact results in the shut down of Cytoplasmic protein synthesis, and this can be restored by simply touching a suitable solid surface. Nuclear metabolism responds to cell shape, however, and is normal only when cells are almost completely spread (Penman et al. 1986). Chondrocytes on the other hand appear to behave in an opposite manner, their phenotype is notoriously unstable and dedifferentiation to fibroblast-like cells is common. The ECM appears to

transmit important positional and mechanical signals to the cytoskeleton, which are transmitted to the nuclear matrix proteins and control the expression of various genes by the chondrocyte. When chondrocytes are cultured in three dimensional suspension systems composed of agarose, collagen gels (Yasui et al. 1982a), and alginate (Hauselmann et al. 1996) they can maintain their normal spherical morphology, and secrete collagen type II. By contrast, in monolayer cultures chondrocytes have a tendency to dedifferentiate as evidenced by changes in both cell shape and biosynthetic activity. Dedifferentiating chondrocytes stop synthesising cartilage matrix components such as collagen type II and are likely to synthesise types I, II, and V collagen (Benya & Shaffer 1982c). Typically, dedifferentiation involves a change from a spherical shape to a flattened fibroblast-like configuration (Shakibaei & De Souza 1997;Shakibaei, De Souza, & Merker 1997). It is clear from these studies that chondrocytes are more likely to maintain their phenotype in three dimensional scaffold cultures which provide a more natural environment for chondrocytes as compared to monolayer cultures.

The mechanisms by which chondrocytes maintain their phenotype in a scaffold system are to a large extent unknown, although, Takigawa et al (1984) have considered an important role of the cytoskeleton in regulating chondrocyte cell shape and phenotypic re-expression (Takigawa et al. 1984). They reported that rabbit costal chondrocytes treated with cytochalasin B change from a polygonal to a rounded shape and synthesise increased amounts of glycosaminoglycans. Colchicine either prevents an increase or inhibits GAG synthesis. Thus the actin in the cytoskeleton may influence continued expression of the cartilage phenotype, as well as initial cartilage differentiation. This work and other more recent findings, suggests that cell shape changes are correlated with changes in chondrogenic function; a round cell shape is associated with differentiation and chondrogenesis, whereas a spread cell configuration is more typical of alternate differentiation states. While it is not always clear which changes come first, it is clear that agents which modify the organisation of the actin cytoskeleton can alter chondrogenic expression.

The influence of extracellular matrix on gene expression has been studied by many workers. It has been shown that proteoglycans and glycosaminoglycans added exogenously stimulate chondrocyte synthesis of sulphated proteoglycans (Bassleer, Rovati, & Franchimont 1998). Conversly, removal of the matrix proteoglycan results in a decreased 35S-sulphate incorporation by the cultures. Thus alteration in the amount of

matrix proteoglycan may affect chondrogenic expression. Although the mechanism of such a response to matrix proteoglycan has not been clarified, it is likely that the effective accumulation of matrix proteoglycans around the chondrocytes in three-dimensional scaffold systems exerts a significant influence on the maintenance of the chondrocyte phenotype. In monolayer cultures, however, almost half of the newly synthesised glycosaminoglycan was released into the medium and led to repetitive loss at each feeding (Kimura et al. 1984). Furthermore, in monolayer culture, contaminating fibroblasts have a tendency to overgrow and chondrocytes with time and serial passage (Guo, Jourdian, & MacCallum 1989).

The use of isolated chondrocytes seeded or encapsulated within artificial extracellular matrices is a relatively recent concept and has been adopted strongly by scientists working in the new field of 'tissue engineering' (Atala et al. 1993;Hauselmann, Masuda, Hunziker, Neidhart, Mok, Michel, & Thonar 1996;Vacanti et al. 1994;Wakitani, Kimura, Hirooka, Ochi, Yoneda, Yasui, Owaki, & Ono 1989). Tissue engineering was defined by Vacanti et al (1994) as ' a science in which the material properties of compounds are manipulated to enable delivery of an aggregate of dissociated cells into a host in a manner that will result in the formation of new functional tissue'.

Three dimensional scaffolds not only provide a more stable phenotype for chondrocytes, there are other potential advantages gained in culturing chondrocytes in-vitro using three-dimensional scaffolds. Chondrocytes obtained from small cartilage biopsies, which are then amplified in monolayer culture in a de-differentiated form, can be re-differentiated in an appropriate biomaterial (Benya & Shaffer 1982b). Scaffolds may provide a solution to practical problems in clinical practice where it is important to have scaffolds of suitable and varying dimensions and shapes that have the correct physical properties for both transportation and implantation.

In order to be able to reconstruct articular surfaces with a biological implant, several other requirements should also be fulfilled. The materials to be used must be biocompatible in such a way that chondrocyte growth in the presence of a normal phenotype should occur. The scaffold must be non-immunogenic, non-antigenic, non-toxic, have a low tissue reactivity and exhibit minimal chronic foreign body reaction. The material should be absorbable after it has served its purpose and the rate of

degradation should be controllable in order to equalise the regeneration rate of the tissue (Freed et al. 1998). By in-vitro and in-vivo testing of different biomaterials it may be possible in the future to discover a material which fulfils all the above criteria.

2.5.2 Materials used in the construction of Scaffold Constructs

Certain substrates are well known to be supportive of cartilage differentiation, for example, culture in and on soft gels (Harada et al. 1990) including agarose (Sun et al. 1986), alginate (Hauselmann, Masuda, Hunziker, Neidhart, Mok, Michel, & Thonar 1996) and collagen (Yasui et al. 1982b). Polymer scaffolds for use in cell transplantation must be highly porous with large surface/ volume ratios to accommodate a large number of cells, and to allow cells to grow into the matrix The following is a brief review of some of the previous and present biomaterials used to construct scaffolds for cartilage tissue engineering.

2.5.2.1 Carbon Fiber

Carbon fibre pad (Medicarb, Dunlop Medical Products, Leyland Medical International Ltd, Lancarshire, England) is an inert material that can be implanted successfully into joints and bone (Minns & Flynn 1978). Woven meshes and carbon fibre rods have been used in the resurfacing of cartilage defects mainly for traumatic lesions, osteochondritis dissecans, and chondromalacia patellae. Although it differs from other forms of scaffold systems in that chondrocytes are not embedded within the material, it does however appear to integrate reasonably well with normal cartilage. As long as particulate debris is not produced, synovitis is not evoked. Histological examination reveals only minimal histiocytic and giant cell reaction. However, as the material is brittle, if the mesh is left in a position in which it is exposed to shearing forces, particulate debris will cause foreign-body reaction and synovitis, leading to joint destruction. The material does not allow cartilage cell attachment and is not particularly supportive of chondrocyte growth. Thus, a common result of such implants is a cartilaginous cap covering a loosely fibrotic carbon mesh that is largely empty of chondrocytes.

2.5.2.2 Collagen Based Scaffolds.

Collagen is the major protein responsible for mechanical strength of tissues throughout

the body. It is the most abundant protein in human beings and animals, constituting 20% to 30% of the total body protein. For the last decade, collagen has been used extensively for its properties as a biomaterial for such purposes as membranes for the artificial kidney, replacement of the vitreous body of the eye, soft tissue augmentation and bone replacement in the mandible (Mannai et al. 1986). Therefore a collagen-based scaffold appears attractive. Indeed, cartilage matrix is made of collagen fibres forming a 3-D network in which proteoglycans and cells are embedded.

Denatured and partially degraded collagen (Gelatin) is used clinically for haemostasis and as a temporary space filler. This material possesses a slight mechanical strength when dry and is jelly-like when wet. Thus, it cannot act as a scaffold for replacing extensive defects in joint surfaces. However, it is very useful for replacing hole-shaped defects (depth at least twice the diameter of the defect) (Holmes, Volz, & Chvapil 1975).

(a) Collagen Gels

Yasui et al (1982) showed that chondrocytes cultured in collagen gels grew three-dimensionally, accumulating extracellular matrix (Yasui, Osawa, Ochi, Nakashima, & Ono 1982a). The exogenous collagen seemed to be incorporated or rearranged into newly formed matrix and served not only as a harmless biologic substance but also as a useful framework in which to produce a new matrix. A biochemical study by Kimura et al indicated that chondrocytes in this culture method maintained their differentiated phenotype during prolonged culture periods (Kimura, Yasui, Ohsawa, & Ono 1984).

Wakitani et al studied the immunological reactions produced by transplanted collagen gel grafts (Wakitani, Kimura, Hirooka, Ochi, Yoneda, Yasui, Owaki, & Ono 1989). Immunological enhancement of peripheral blood mononuclear cells was analysed by direct and indirect blastformation reactions, which are generally thought to be sensitive enough to detect the occurrence of an immunological response. Neither chondrocytes or collagen used in the experimental system were immunogenic enough to induce direct blastformation reactions, nor even the indirect blastformation reaction induced by pokeweed mitogen. It was concluded that the collagen gels did not have any significant immunogenecity.

(b) Collagen Sponge

Very few studies have been performed using a collagen sponge as a scaffold material for chondrocytes. It is a felt-like material derived from bovine achilles tendons and therefore consists of type I collagen. Outside the field of tissue engineering it is widely used as a haemostat during surgery. Helistat (Colla-Tec, Inc. Plainsboro, USA) is a rectangular, flat, soft, white, pliable sponge of varying dimensions. The sponge is sterilised using ethylene oxide cycle and blister packed to maintain its sterility.

The manufacturing process is proprietary and involves a processing stage in which collagen fibres are freed from tendon matrix and purified by the removal of non-collagenous proteins. Following chemical treatment at controlled pHs the collagen is dispersed in non-pyrogenic water to form a uniform gel-like liquid in which the individual fibrils of collagen are highly swollen. The dispersate is then lyophilised and cross linked to stabilise it. The sponges are cut to size and sterilised with ethylene oxide prior to packaging in blister-packs (Colla-Tec information).

The collagen sponge is extremely hydrophilic and one gram can absorb up to 40-60g of water which can be increased by increasing the degree of crosslinking. The hydrophilic nature of the sponge may enhance its ability to support cell growth and tissue repair (Speer et al. 1979). Under mechanical compression the sponge easily deforms but regains its original shape and size after resorption of water. The collagen sponge is biodegradable and the time taken for degradation in the body depends on the degree of crosslinking. Higher degrees of cross linking results in a slower absorption by the body.

Living cells generally have negatively charged cell membranes and therefore prefer an oppositely charged surface for attachment. They also prefer a microscopically rough surface. The collagen sponge satisfies both criteria in that it is negatively charged and due to the high content of both diamino- and dicarboxylic amino acids with the presence of carbohydrate moieties it has a rough surface at the molecular level. Under the electron microscope, the surface of the sponge is uneven and irregular showing pores varying in size from $50\text{-}159 \times 10^{-3}$mm.

The collagen sponge has been used in a number of clinical applications including as an artificial skin (Abramo & Viola 1992), vaginal barrier contraceptive, dural graft in

neurosurgery (Narotam, van Dellen, & Bhoola 1995), wound dressing (Chvapil, Chvapil, & Owen 1986) and surgical haemostat. Most clinical studies show the collagen sponge to be biocompatible with very little rejection type immune response and foreign body reaction (Remberger & Hubner 1979). When used in burns patients, histological analysis revealed extensive penetration of fibroblasts, macrophages and polymorphonuclear lymphocytes (Abramo & Viola 1992). This observation enhances the possibility that the collagen sponge should allow adequate ingrowth of chondrocytes if used to repair cartilage defects. When implanted into animal organs there was a normal local inflammatory response characterised by mononuclear phagocytic cells resulting in a gradual degradation and absorption of the sponge over a nine week period (Cutright et al. 1973). The most rapid absorption occurred in the lungs. Very few studies have been performed using the collagen sponge as a matrix scaffold for chondrocytes in the repair of articular cartilage defects. A study in rabbits using collagen sponge to repair osteochondral defects demonstrated macroscopically and histologically compliant repair tissue at 44 weeks after implantation and the sponge allowed ingrowth of chondrocytes. The authors concluded that the successful results using the sponge were due to its porosity, biodegradability, biocompatibilty and its ability to mechanically protect cells and newly formed matrix (Speers, Birdwell, & Dixon 1979). Unfortunately, the collagen sponge was not cell-seeded in these experiments which may have reduced its potential for a much better repair.

There is evidence that the collagen sponge may actually enhance chondrosynthesis (Matsuda et al. 1995)and if impregnated with certain growth factors prior to implantation it may greatly accelerate the repair response. Basic fibroblast growth factor impregnated collagen sponges seeded with chondrocytes were shown to be successful in providing a suitable scaffold for cells to proliferate, express their distinct phenotype and mature quickly when implanted subcutaneously (Fujisato et al. 1996).

The most successful use of the collagen sponge was reported recently in the Journal of Bone and Joint Surgery. Collagen sponge seeded with chondrocytes was implanted into rabbit knee articular cartilage defects. To prevent the ingrowth of fibroblasts and immune response cells from the vascular system the collagen sponge was coated with a non-permeable barrier on the surface opposing subchondral bone. Follow up histological analysis demonstrated remarkable results showing large amounts of newly formed cartilage which biochemically closely resembled articular hyaline cartilage.

The safety and efficacy of Helistat has been investigated fully by the manufacturer and it appears to be a safe material for in vivo use. With recent successes in regenerating cartilage implanted into defects, it has huge potential for further study and clinical use.

2.5.2.3 Alginate

Background and Manufacture

Because of their affinity for water, their total lack of toxicity, and the ability to form viscous solutions and gels, alginates have long been widely used in the food industry. More recently, their ability to form gels very rapidly in the presence of calcium ions under extremely mild conditions has been exploited for immobilisation of particulate enzymes and many different kinds of living cells including chondrocytes (Mok et al. 1994).

Alginates are mucopolysaccharides extracted from seaweed. A large industry has evolved around the cultivation of algae due to the widespread uses of alginates. Macrocystis pyrifera, the brown seaweed is the main source of algin and has growth characteristics making it an ideal raw material for modern technology. It grows in relatively calm waters and in large, dense beads. The plant is perennial and thus can be harvested on a continuing basis, its rapid growth permits up to four cuttings per year.

Alginate forms between 14% and 40% of the dry weight of brown algae. It is located in the intercellular matrix as a gel containing sodium, calcium, magnesium, strontium and barium ions and functions as a structure forming component giving the algae both mechanical strength and flexibility. The commercial methods of producing alginate from seaweed are proprietary. In order to isolate alginic acid from seaweed, the seaweed is washed, milled and reduced to powder. It is treated with hot water and alkali in order to produce a solution of laminaran, fucoid and algin. Dilute acid is then used to eliminate acid soluble substances, while converting alginate to free alginic acid. The alginic acid is treated with a strong solution of a sodium salt, such as sodium carbonate to produce sodium alginate.

Use of alginates in the food industry include water retention, gelation, emulsification, a thickening agent, film formation and as a suspension agent.

Structure and Properties

Alginate is composed of linear chained molecules of (1-4)-linked residues of (-D-mannuronic acid (M) and (-L-guluronic acid (G) in different proportions and sequential arrangements. The most common arrangement is that of a block co-polymer, in which long, homopolymeric sequences of ManA residues (MM-blocks) and similar sequences of GulA residues (GG-blocks) are interspersed between sequences of mixed composition (MG-blocks) (Malissard et al. 1993). The polymannuronic acid chains are flat, ribbon like, molecules which are internally stabilised by hydrogen bonds. The structure of the polyguluronic acid chains is slightly different and they are buckle ribbon like shaped molecules. It has recently been reported that some relevant properties of calcium alginate gels, including mechanical rigidity, swelling and shrinking characteristics, and resistance to interference by monovalent cation, were highly dependent upon the molecular weight of the alginate, its ManA/GulA ratio, and especially its content of GG-blocks. The higher the guluronic acid content, the stiffer the gel.

When sodium alginate solution is added to divalent metal ion solutions such as calcium, an alignment of the G-block regions occurs and the metal ions are co-operatively bound between two aligned chains like eggs in a box. Thus the conformation is known as the 'egg box model' (Gregor T.Grant et al. 1973). It is thought that gelation of calcium alginate occurs when there is co-operative association of polyguluronate sequences of twenty or more residue. Although any divalent cation may be used for the gelation process, calcium is the most commonly used due to its relatively mild effects on cells. The following equation represents the reaction which occurs when divalent metal ions (M++) react with sodium alginate ;

$$2Na(Alg) + M^{++} = M(Alg)_2 + 2Na^{+}$$

The mechanical properties of alginate gels are highly dependant on the molecular weight of the alginate used. The higher the molecular weight the stiffer the final alginate gel construct. The number of crosslinks in an alginate gel is dependant on the segment concentration of GG-blocks. Therefore the greater the number of GG-blocks, the greater the cross link numbers and the stiffer the final gel construct.

Viscosity is a measure of the internal friction or resistance to flow of two adjacent fluid layers. It is related closely to the molecular weight of alginate. Alginate preparations vary from low viscosity alginates with molecular weight between 12,000 to 80,000 and high viscosity alginates with molecular weight between 120,000 to 190,000. These properties play an important role in cell cultures as they affect the diffusion of nutrients into alginate gels and also possibly affect chondrocyte matrix synthesis and chondrocyte cell proliferation.

Biocompatibility

Certain shellfish contain specific enzymes that digest alginate (alginases) in order to facilitate digestion of algae and some bacteria which have an outer slime coat containing alginate. Humans, however, do not synthesise alginases and its breakdown occurs via enzymes contained in the vacuoles of polymorphs and macrophages.

Alginate is commonly used in the food industry as a food texturiser (Sandberg et al. 1994). Although very few long term studies have been performed assessing the effects of alginate on the gastrointestinal tract, experimental data from animal studies reveals it to be safe for up to 2 generations of offspring. Calcium alginate has also been considered for use as a carrier of salicylic acid into the colon of patients with ulcerative colitis (Lin & Ayres 1992) and also has been suggested as a delivery device for therapeutic compounds (Narayani & Rao 1995).

The other large area of use of calcium alginate is in wound dressings. In partial thickness and full thickness wounds in which alginate dressings have been applied, biopsies taken at fourteen days, showed complete filling of the wounds with connective tissue and degradation of calcium alginate. However, recent studies have shown that a foreign body (fibrous) reaction occurs after alginate capsules are implanted into wounds (Lansdown & Payne 1994a). Further recent studies have indicated that alginate has a cytopathic effect against fibroblasts and a foreign body reaction also occurs when alginate is implanted subcutaneously in mice (Suzuki et al. 1998). This response appears to be species dependant with a more aggressive inflammatory reaction in rats as compared to pigs and humans. Despite the slight immune reaction, alginate dressings in burns patients and diabetics with ulcers are successful at epidermal cell regeneration as they maintain moist conditions in the wound which promotes healing (Lansdown & Payne 1994b).

Otterlei et al demonstrated that the mannuronic acid component of the alginate stimulates monocytes to produce high levels of cytokines and tumour necrosis factor and is the most likely agent responsible for the immune response against alginate (Otterlei et al. 1991). However, it has been shown by other workers that crude alginate incites a greater mitogenic activity in lymphocytes than does purified alginate and thus the immune reaction may be due to non-specific impurities such as phenolics found in crude alginate preparations. They could not confirm the relationship of mannuronic acid content to mitogenic activity in lymphocytes.

Most in-vivo studies have found that calcium alginate eventually becomes absorbed by the body. The rate of absorption depends on the region of the body in which the implants are situated. Lansdown and Payne (Lansdown & Payne 1994c) found remnants of calcium alginate 3 months after subcutaneuos implantation in rats whereas others have reported complete absorption at 6 weeks post implantation into the small bowel mesentery of rats.

Applications in cartilage tissue engineering

Calcium alginate gels have been used successfully to culture chondrocytes in vitro. Guo et al have shown that chondrocytes encapsulated within calcium alginate gels can proliferate in tissue culture, although at a rate approximately 30 per cent of similar cells placed onto tissue culture plastic (Guo, Jourdian, & MacCallum 1989). Hauselmann et al have demonstrated production of a matrix rich in collagens and proteoglycans. Their study also demonstrated that bovine articular chondrocytes remain metabolically active for 8 months when cultured in alginate beads (Hauselmann et al. 1994). The cells were shown to have retained the ability to synthesise cartilage specific molecules such as keratan suphate-bearing aggrecan and type II collagen. In addition to collagen type II, the cells also synthesised type IX collagen, which plays an important role in crosslinking of the collagen in network in articular cartilage (Eyre, Wu, & Apone 1987;Eyre, Wu, & Woods 1998), but no type X collagen, a marker for hypertrophic cartilage undergoing endochondral ossification (Linsenmayer et al. 1998) and for articular chondrocytes that have become hypertrophic in osteoarthritis (von der Mark et al. 1995).

Hauselmann et al carried out further analysis on the matrix formed by adult human chondrocytes cultured in alginate gel (Hauselmann, Masuda, Hunziker, Neidhart, Mok, Michel, & Thonar 1996). They found it to be composed of two compartments: a thin rim of cell associated matrix that corresponds to the pericellular and territorial matrix of articular cartilage and a more abundant further-removed matrix, the equivalent of the interterritorial matrix in the tissue. On day 30 of culture, the relative and absolute volumes occupied by the cells and each of the matrix compartments in the alginate constructs were nearly identical to those in native articular cartilage . Furthermore, the concentration of aggrecan in the cell associated matrix was similar to that in adult human articular cartilage and was about 40 fold higher than in the further removed matrix compartment.

In a direct comparison between collagen and alginate gel to culture chondrocytes, Van Susante et al concluded that collagen gels showed a significant increase in cell numbers, but the chondrocytes dedifferentiated into fibroblast-like cells from 6 days onwards (van Susante et al. 1995). In alginate gels, initial cell loss was found, but the cells maintained their typical phenotype. Although the total quantity of proteoglycans initially synthesised per cell in collagen gel was significantly higher, expressed per cell, the quantity in alginate gel eventually surpassed collagen. It was suggested that the initial cell loss in alginate may have been due to cell death on exposure to high Ca^{2+} concentrations during gelation.

When subcutaneously implanted into mice calcium alginate constructs containing chondrocytes were histologically demonstrated to contain matrix products with a cartilage type appearance. The histological examination of the chondrocyte alginate constructs demonstrated basophilic nodules and lighter staining internodular spaces consistent with concentric deposition of extracellular matrix from isolated chondrocytes. Furthermore, mechanically, the constructs containing chondrocytes were able to sustain higher loads than calcium alginate without cells (Paige et al. 1996). This study represents the possibility of using alginate as a three dimensional construct for cartilage tissue engineering. The ability to mold calcium alginate gels to produce defined shapes creates the opportunity to fabricate custom-designed cartilage implants.

An injectable biodegradable polymer as a delivery vehicle for chondrocytes may be more useful than a solid pre-formed construct particularly when one considers the

nature of articular cartilage defects. Atala et al have demonstrated the successful implantation of alginate-chondrocyte gel into mice by a method of injection (Atala, Cima, Kim, Paige, Vacanti, Retik, & Vacanti 1993). Gross examination of the injection sites with increasing time showed that the polymer gels were progressively replaced by cartilage. The ultimate size of the cartilage formed was related to the initial chondrocyte concentration injected, and appeared to be uniform and stable. Histological analysis of distant organs showed no evidence of cartilage or gel migration, or granuloma formation. Although, the work was carried out on mice for the treatment of vesico-ureteric reflux, there is no reason why this technology could not be employed in cartilage tissue engineering. They concluded that chondrocyte-alginate suspensions are injectable, appear to be non-migratory and able to conserve their volume.

Recently there has been much interest in using mesenchymal stem cells as precursors for chondrocytes and osteoblasts. By subjecting these cells to specific conditions, they are known to differentiate to chondrocytes. Alginate has proved useful as an in vitro model used for the cultivation of chondrocytes (Shakibaei & De Souza 1997). Isolated mesenchymal cells were grown in alginate for up to four weeks. A sub-population of the cells differentiated to chondrocytes and exhibited a stable phenotype until the end of the culture period. After 3 to 4 days a cartilage-specific matrix started to develop. Fibroblast-like cells from this mixed culture did not survive and became necrotic. When alginate was later dissolved by chelating agents, only chondrocytes were isolated. This pioneering work has raised possibilities of obtaining pure chondrocyte cultures from mesenchymal stem cells and the potential to resuspend the chondrocytes in alginate gel for in vivo implantation.

2.5.2.4 Agarose

Background and Manufacture (Wieme et al 1965) (Wieme 1965)

Agar is a polysaccharide extracted from various red algae. It si derived from the cell membrane. The algae (eg Gelidium corneum and Gelidium amansii) are harvested in summer and bleached on the shore. They are further treated in winter. After boiling in water acidified with sulphuric acid or acetic acid, an extract is obtained which is filtered and collected in shallow wooden containers. A gel is formed which is frozen and thawed exuding water and soluble impurities. The raw product resulting from this

preparation differs widely according to the season in which algae were collected, the kind of algae used and the subsequent treatment of the raw material. Agar is largely used in the food-processing industry since it forms stable viscous solutions at low concentration and is very resistant to bacterial attack. The chief producers are Japan and, to a lesser extent, Australia and the United States.

Properties

The commercially available powder that is called agar, is poorly defined from the chemical point of view. Analysis reveals varying content in cations (especially Ca^{2+} and Mg^{2+}) and the presence of several carbohydrates (mainly D-galactose and 3,6-anhydro-L-galactose; also a small percentage of D-glucuronic acid and L-galactose). Pyruvic acid and sulphate can also be demonstrated.

Agar powder is a mixture of at least two polysaccharides. They can be easily separated: upon acetylation, only one of them is soluble in chloroform. Thus the agarose acetate (soluble) and an agaropectin acetate are differentiated; upon saponification they respectively regenerate agarose and agaropectin. The situation is rather similar to that of starch, which separates into amylose and amylopectin.

Agarose has the following empirical formula : $[C_{12}H_{14}O_5(OH)_4]_n$, corresponding to a disaccharide. This agarobiose is composed of β-D-galactopyranose and 3,6-anhydro-L-galactose.

Agarose is built up from agarobiose units linked to form a linear galactan. Along the linear structure some OH groups may be involved in a semi-ester linkage with sulphate, but sulphate does not constitute an essential component of the molecule. It was originally thought that the gelification of agarose was due to the sulphate groups together with calcium ions. It is now accepted that hydrogen bonding is responsible for the gelification.

Agarose forms a gel matrix that is nearly ideal for diffusion and electrokinetic movement of biopolymers. Therefore it is useful for electrophoresis, immunodiffusion and artificial scaffold matrices. The sulphate content and gel strength can be varied according to the requirements. The anionic groups in an agarose gel are affixed to the

matrix and thus restrained from such movement. However, dissociable cations and their hydration spheres can migrate freely within the gel (Data Sigma chemicals).

Applications in cartilage tissue engineering

Horwitz and Dorfman demonstrated the possibility of growing chondrocytes on soft agar gel (Horwitz & Dorfman 1970). A chondrocyte monolayer was quickly established with evidence of dedeifferentiation to fibroblast-like cells becoming anchorage dependent. A major advance was achieved when it became possible to establish chondrocyte cultures in gel matrices. Yasui et al and Gibson et al cultured embryonic chick chondrocytes in collagen (Gibson et al. 1983;Yasui, Osawa, Ochi, Nakashima, & Ono 1982a). Benya and Schaffer (1982) and Bounelis and Daniel (1983) used agarose as an inert matrix to grow chondrocytes from articular cartilage from 8-week-old rabbits and chick sternum, respectively (Benya & Shaffer 1982a;Bounelis & Daniel 1983). The use of agarose rather than soft agar offers a sulphate free, biologically inert three dimensional matrix. The negatively charged matrix of agar is thought to depress collagen synthesis by mimicking proteoglycans and therefore agarose, which is electrically neutral is a more useful material for scaffold construction. Since agarose, like other scaffold gels, retards diffusion of macromolecules, newly-secreted matrix components accumulate close to the cell surface to a greater extent than in liquid media and monolayer cultures. As a result, chondrocytes establish a differentiated phenotype and synthesise considerably more collagen and proteoglycans than monolayer cultures.

The culture conditions in agarose allow chondrocytes to develop in a manner consistent with conditions prevailing in articular cartilage in vivo. The cells distribute in agarose gel in a typical arrangement, forming a pericellular region which is surrounded by an interterritorial-like region. The agarose gel forms a diffusion pathway, comparable to the cartilage matrix, across which nutrients and metabolites pass to and from the chondrocytes (Delbruck et al. 1986). It is interesting to note that fibroblats obtained from surrounding joint tissue cannot be grown in the agarose gel culture under conditions which allow growth of chondrocytes. Agarose therefore permits chondrocyte selection.

The chondrocytes growing in agarose display features of actively synthesizing cells. Their rough surfaced endoplasmic reticulum is prominent, the vacuoles of the golgi

apparatus maturing surface are filled by particles and filaments both visible in the pericellular region as well. These features, which have been repeatedly described in studies on hyaline cartilage suggest, that from a morphological point of view the components synthesised by chondrocytes growing in agarose are similar to those produced by chondrocytes in situ.

In agarose, there is a continuous increase in the rate (μg/ml/day) of glycosaminoglycan accumulation in culture medium for up to 50 days, whereas in monolayers an increase in the rate of accumulation occurs initially, but this is followed by a time dependent decrease after the first 8-10 days (Spirito, Goldberg, & Di Pasquale 1988). The increase in dermatan sulphate content of secreted glycosaminoglycans is a common feature of human chondrocytes in monolayer cultures (Oegema, Jr. & Thompson, Jr. 1981). This is not a feature of extracellular matrix in vivo as demonstrated by Von der Marke et al (von der Mark, von der Mark, & Gay 1976)and neither is it a feature of human chondrocytes cultured in agarose as demonstrated by Delbruck et al (Delbruck, Dresow, Gurr, Reale, & Schroder 1986). In monolayer cultures the corresponding proteoglycan molecules become smaller, contain only a few chondroitin sulphate chains, a relative higher proportion of dermatan sulphate chains and no longer bind to hyaluronic acid (Ho, Levitt, & Dorfman 1977;Okayama, Pacifici, & Holtzer 1976;West et al. 1979). In parallel with this, the rate of hyaluronate synthesis increases. In Agarose cultures, the hyaluronate content of the extracellular matrix remains comparative to the hyaluronate content of articular cartilage matrix (Delbruck, Dresow, Gurr, Reale, & Schroder 1986).

Ostensen et al (1991) investigated the use of agarose in growing human articular cartilage chondrocytes from Rheumatoid arthritis and Juvenile Rheumatoid arthritis affected patients (Ostensen et al. 1991). Arthritic chondrocytes proliferated and synthesised extracellular matrix. However compared to healthy controls, proliferation and formation of extracellular matrix was delayed in chondrocytes from actively inflamed joints. During several weeks of culture, the observed differences between rheumatoid patients and adult controls regarding proliferation and proteoglycan synthesis levelled off except in cases of end-stage arthritis. They concluded that the agarose culture system is a suitable in vitro tool for further studies on human joint disease.

Articular cartilage in the adult is a stratified tissue in which both the morphology of the chondrocytes and the biochemical composition of the surrounding extracellular matrix show characteristic variations with distance from the surface of the tissue. Aydelotte and Kuettner (1988) were able to reproduce these differences by culturing chondrocytes in agarose (Aydelotte & Kuettner 1988). They demonstrated differences in the morphology and metabolism of cells harvested from superficial and deep regions of articular cartilage. Cells from the superficial zone of calf and adult bovine metacarpophalangeal cartilage were shown to have a more irregular shape, to divide more slowly, to synthesise proteoglycans that were smaller and less able to form aggregates, and to produce larger collagen fibrils chondrocytes from deep zones. Again agarose was found to provide a satisfactory in vitro culture system for the study of chondrocyte properties.

The agarose culture system is tranparent and allows the study of colony formation in reponse to changes in environmental conditions ie growth hormone etc. It also allows the study of progression of cell size over the culture period and the imaging of cell shape changes produced by mechanical deformation of the gel (Steinmeyer, Ackermann, & Raiss 1997;Steinmeyer & Knue 1997). Buschmann et al (1995) studied the mechanical properties of agarose chondrocyte constructs and compared these with native articular cartilage (Buschmann et al. 1995). Dynamic stiffness and compression induced streaming potential in the seeded gels were larger than in gels without cells after one week of culture. By one month, the dynamic stiffness and streaming potential were about one-fourth that of native calf articular cartilage, consistent with a GAG density and cell density of approximately one-fourth that of native tissue. They concluded that chondrocytes cultured in agarose gel provide a useful model system to study the mechanisms by which chondrocytes respond to mechanical compression in native cartilage. In addition they confirmed preservation of phenotype and continued synthesis of matrix components, matrix assembly and deposition is accomplished in a mechanically functional manner in agarose culture. Furthermore, at 41 days of culture, they found that proteoglycan and protein synthesis in chondrocyte agarose discs can be altered by static and dynamic compression in a manner similar to that observed in native cartilage explants. Thus, increasing levels of static compression decreased biosynthesis in a dose dependent fashion, while dynamic (sinusoidal) compression at high frequencies (0.01 and 0.1 Hz) could stimulate biosynthesis, trends similar to those of native articular cartilage.

The extraordinary properties of agarose to allow chondrocyte phenotype stability and allow satisfactory study of mechanical influences on chondrocytes combined with the ability to visualise the individual cells could facilitate the study of mechanisms at a single cell level by enabling quantification of cell deformation and possibly, membrane mediated events. It may be possible to perturb matrix assembly through the addition or removal of a particular matrix constituent and to subsequently determine the mechanical significance of the constituent (Buschmann, Gluzband, Grodzinsky, & Hunziker 1995).

The remarkable ability of agarose to provide a suitable matrix environment for chondrocyte proliferation and phenotype stability has prompted in vivo work. Rahfoth et al used agarose-chondrocyte constructs for repair of articular cartilage defects in rabbits. They found that in nearly 50% of cases the chondrocyte implants gave rise to a repair cartilage similar to hyaline morphology and an extracellular matrix containing type II collagen and proteoglycans. In about 25% of cases even nearly perfect cartilage reconstruction was achieved (Rahfoth et al. 1998). Most of the grafts integrated with the adjacent cartilage above the tidemark and with the subchondral bone, but never with the calcified cartilage. At 18 months post implantation the essential features of hyaline cartilage were preserved and in some cases were improved with time. Furthermore, there were no signs of graft-versus-host rejections or infiltration by immune cells, although immunological parameters including antibodies against agarose were not determined.

The transplantation of chondrocytes embedded in agarose represents an alternative to other systems and will surely encourage the further development of this method.

2.6 Bioreactor Culture Systems

Articular cartilage with its relatively low cell concentration compared to other tissues has remarkable properties that are considerably influenced by the matrix and are extremely difficult to reproduce in artificial scaffolds. The following is a brief review of the biophysical properties of cartilage (Maroudas & Schneiderman 1987).

2.6.1 Biophysical properties of cartilage

The main functions of cartilage are to distribute load over the subchondral bone and to provide a low friction and low wear surface with suitable lubricating properties. These functions depend on the high water content of the cartilage matrix, allied to a low hydraulic permeability and a high swelling pressure. It is the high glycosaminoglycan content and its organisation in a three dimensional architecture which confers these properties on cartilage.

Glycosaminoglycans by the virtue of their acidic groups, carboxyl and sulphate are negatively charged, which accounts completely for the resultant negative fixed charge density in cartilage at physiological pH's. The effective charge content of the pericellular matrix of chondrocytes has been determined while the matrix is being synthesised by cells grown in culture for several weeks. It was concluded in this study that the marked difference in the availability of negative groups between chondrocytes cultured from articular and non-articular cartilages might reflect differences in the interaction of these negative groups with matrix components. These differences may be responsible for the distinct structural organisation of these two cartilaginous tissues which possess different mechanical functions (Van Damme et al. 1997). The negative charge of matrix not only influences the structural organisation of cartilage but also effects the diffusion of solutes through cartilage. Anions would be expected to move in a different manner to cations resulting in an unequal distribution of solutes within the matrix. Work by van den Berg et al highlights the importance of the negative charge and suggests that the negative charge of cartilage matrix may be responsible for the low penetration of immune complex components into cartilage and may explain the tolerance of cartilage to host immune responses(van den Berg et al. 1986).

The rate of movement of solutes from external solution into cartilage and vice versa is governed by three factors (a) the resistance of a stagnant liquid film at the cartilage-fluid interface which in life is the interface between cartilage and synovial fluid (b) the distribution coefficient of a solute between cartilage and external solution and (c) the effective diffusion coefficient of the solute in cartilage. It has been shown that provided the liquid in contact with cartilage is sufficiently stirred, the latter factor will be dominant (Maroudas 1975b).

Small **uncharged** solutes such as urea, glucose, glycine and proline that are of smaller size than the cartilage pores behave in cartilage as in free solution. The diffusion coefficients of these solutes in cartilage are equal to about 40-50% of their values in aqueous solution. The reduction in the diffusivity in cartilage can be explained by two factors; 1) a smaller effective area being available to diffusion because of the solid matrix, and 2) a more tortuous path (Maroudas 1975a). Despite the inhibitory effect of cartilage matrix, it has been estimated from permeability data for glucose that chondrocytes even in the thickest cartilage, eg in the patella where it can reach a depth of 5mm, obtain enough glucose from the synovial cavity to supply adequate nutrition. The same is true of other small solutes such as oxygen or the sulphate ion.

Small electrically **charged** solutes have slight restrictions in their distribution in cartilage. Cations have partition coefficients higher than unity, increasing with increasing glycosaminoglycan content. Anions, on the other hand, have partition coefficients less than unity, which decrease as fixed charge density increases. Ions such as Na^+ and Cl^- are freely distributed and well dispersed in cartilage matrix and make a contribution to the ionic component of the overall swelling pressure of cartilage. The divalent calcium ion on the other hand forms strong interactions with the negatively charged groups in cartilage and thus cartilage can tolerate much higher concentrations of calcium without precipitation of calcium orthophosphate than would be possible in aqueous solution. The role of proteoglycans in the control of calcification might thus be to concentrate the calcium in the matrix, initially without precipitation of calcium phosphate. Once the proteoglycans are lost from the matrix, calcification would be established.

Whilst small solutes are able to diffuse freely in and out of cartilage under all physiological conditions, the passage of **larger molecules** is very restricted because of steric exclusion effects exerted by the glycosaminoglycan molecules. The diffusibility of solutes of the size of for example serum albumin is extremely sensitive to variations in the glycosaminoglycan content. For a two-fold increase in negative fixed charge density, there is a tenfold decrease in the diffusivity of serum albumin. However, for larger solutes this relationship breaks down. The reason for this is unclear. It is possible that there are a small fraction of "pores" in cartilage that are relatively larger than the rest and therefore allow the transport of predominantly large molecules. The diffusion of large solutes is then not limited by the size of the smaller pores and the relationship

of pore size and diffusion breaks down. Such a view seems to be consistent with the relatively high diffusion coefficient of serum albumin, which has a relatively high diffusivity for its size. If serum albumin molecules were moving through spaces only slightly larger than themselves, one would expect a frictional retardation and hence a considerable lowering of diffusivity.

The existence of a small proportion of channels which allow the passage of solutes of high molecular weights (above 100,000) may explain the reason why proteoglycan fragments of relatively high molecular weight are able to diffuse very slowly out of cartilage in the course of normal matrix turnover. Unlike the case of small solutes, there could be conditions under which the utilisation of large solutes by the chondrocytes might be limited by their rate of diffusion through the matrix. For example, the variations in glycosaminoglycan content between different joints and between different zones of the same joint leads to considerable differences in the penetration of antibodies. If auto-immune destruction of cartilage is involved in rheumatoid arthritis, then the fact, for instance, that the femoral head is relatively less prone to this disease than more peripheral joints might be explicable in terms of its higher glycosaminoglycan content.

It has often been suggested in the past that cartilage may obtain its nutrients from the action a physiological "pump" by which the compression of cartilage surface under loads leads to expression of fluid whilst the subsequent relaxation is accompanied by the reabsorption of fresh fluid with solutes from the synovial cavity. However, work by Maroudas et al (1987) shows that although the mechanism is valid for large molecules with low diffusivities, this is not the case with small solutes such as glucose where a pump mechanism would only account for 20% of the diffusion of glucose into cartilage (Maroudas & Schneiderman 1987).

In summary, cartilage matrix is a tissue of high water content, high negative fixed charge density and very fine pore size. Its fixed charge density is responsible for its selective permeability to ionic solutes and its high osmotic potential. The combination of high water content allied to a fine pore size results in the high permeability of cartilage to small solutes coupled to an almost complete exclusion of large molecules. This means that whilst the smaller nutrients and metabolites are able to diffuse freely in and out of cartilage under all conditions, the passage of large molecules such as

antibodies or glycosaminoglycan fragments is restricted and highly dependent on local variations in the proteoglycan content.

2.6.2 The need for Bioreactors

The unique properties of articular cartilage are not confined to matrix alone, chondrocytes too have properties which need to be addressed carefully in the design of scaffolds and culture systems. In biomedical research, the metabolic activity of cultured cells must be monitored and their environment optimally adjusted to promote long-term maintenance of viability and high cell density. Oxygen tension in the developing growth plate of articular joints is thought to be negatively correlated with vascularity and provides a good example of the importance of maintaining optimal environmental conditions. The lack of oxygen in the hypertrophic zone is deemed necessary for hypoxia-related modulation of chondrocyte metabolism that results in calcification (Shapiro et al. 1983). Oxygen tensions of 5% in embryonic mesenchymal cells will potentiate the chondrocyte phenotype, whereas higher oxygen tensions will potentiate the osteoblast phenotype. These requirements necessitate accurate regulation of environmental conditions, well-balanced transport of nutrients to the cultured cells and the efficient withdrawal of toxic wastes and metabolic byproducts.

The phenotypic instability of monolayer cultures has led researchers to utilise three dimensional culture systems such as agarose, alginate, collagen gels and fibrous polyglycolic acid cultures to study the regulations of collagen and proteoglycan with regard to chondrocyte phenotype. From the above review, it is clear that the physicochemical characteristics of artificial matrices can have an effect on the chondrocytes entrapped within them. Most artificial scaffold systems although biocompatible and possessing the ability to support chondrocyte growth suffer from problems of solute diffusion and electrical charge distribution which makes the selection of suitable materials extremely difficult.

In the case of normal articular cartilage the pore size is a critical parameter in selecting an appropriate matrix material. Pore sizes must not only allow sufficient diffusion of low molecular weight substrates into the matrix and waste product diffusion out of the matrix, they must also prevent the wholesale leakage of enzymes, proteoglycans, growth promoting hormones and whole cells out of the matrix. Thus, larger pore sizes

which may appear attractive at improving the mass transfer of nutrients to embedded chondrocytes may in practice encourage excessive loss of important signalling and growth promoting chemicals. In association with a reduction in material density, larger pore sizes may also result in a mechanically poor construct. This would obviously have implications in clinical practice, as it would not be able to support normal loading. Furthermore, the electrical charge of scaffold matrices may also be an important factor in a similar manner to the negative fixed charge of articular cartilage glycosaminoglycans which is implicated in the distribution of solutes and possibly chondrocyte behaviour.

To date only a handful of biomaterials have been shown to successfully allow the proliferation of chondrocytes and these include agarose, alginate, collagen and polyglycolic acid gels. The diffusion characteristics of alginates, for example, have been extensively investigated (Amsden & Turner 1999) and it has been found that they are suitable for cell culture because they allow free diffusion of small substrates with limited leakage of larger substrates. Although these materials fulfil some of the requirments for a suitable environment for chondrocyte culture, they still suffer from poor mass transfer of nutrients to embedded cells (Freed, Marquis, Nohria, Emmanual, Mikos, & Langer 1993;Freed, Vunjak-Novakovic, & Langer 1993). This results in slow proliferation rates of chondrocytes and a substantial decline of matrix production with time (Baker & Goodwin 1997).

An important avenue to solving some of the problems with the above scaffold materials may lie, not in making the scaffold materials more porous, but by the use of dynamic culture systems to improve mass transfer of nutrients. Until recently, most researchers have used static culture systems for the culturing of chondrocytes, both in monolayer and in three dimensional scaffold cultures. Freed et al (1993) showed that the environment used for cell seeding and tissue culture contributed to the composition of the resulting cartilaginous constructs (Freed, Vunjak-Novakovic, & Langer 1993). In particular, constructs grown under mixed conditions were larger and contained more cells and extracellular matrix than constructs grown statically.

The potential to, not only culture chondrocytes in three dimensional constructs, but to also enhance proliferation rates is important for tissue engineering as it allows small biopsy specimens to be used to create larger specimens which may serve useful for

implantation. High final cell densities are essential to regenerate compact, functional cartilage tissue and are also known to help maintain the differentiated phenotype (Bruckner et al. 1989), probably via the "community effect" (Gurdon 1988). Furthermore, high cell densities are associated with high glycosaminoglycan and collagen contents which ensure that the scaffolds become more stiff and show less deformity to compressive strain. Due to the inevitable loss of some cells during the implantation procedure and cell death prior to acclimatisation in the new in-vivo environment, it is essential to start off with a high cell density.

Compared to monolayer cultures which can support a maximum of 1 million cells/ml. Three dimensional cultures in tissue engineering can lead to much higher cell densities, under the optimal conditions, similar to the native tissue. Human articular cartilage normally contains about 15 x 10^6 cells/ml. Such cell densities require very frequent changes of medium to maintain stable nutrient conditions (Sittinger et al. 1996). The use of perfusion culture chambers would facilitate a stable pH and constant concentration of nutrients such as glucose. Culture handling would be easier and risk of infection greatly reduced. This is especially important for prolonged cultures up to several weeks or months. It would suggest that static three dimensional culture systems are not sufficient to meet the demands of the complex culture conditions required for advanced tissue engineering. Cell densities in a gel matrix culture under static conditions are severely limited by diffusion of nutrients and waste products through the matrix. This limits the number of cells that can be grown in a three dimensional construct.

2.6.3 Bioreactor Design

A bioreactor is defined as any device that provides the transport system for nutrients to cultured cells and allows the efficient withdrawal of toxic wastes and inhibitory metabolic byproducts. The designs of bioreactors vary from simple types which perfuse medium automatically to complex designs which offer medium circulation, monitoring environmental conditions and cell retrieval.

The use of bioreactors for tissue engineering could offer several potential advantages as compared to static systems including :

1) uniform and efficient mixing coupled with precise control over mass transfer rates
2) regulation of shear stress within the vessel
3) maintenance of constant pH, gas partial pressures (pO_2, pCO_2) and nutrient levels (eg, glucose)
4) process control strategies which can match the changing needs of a growing implant over the entire duration of its cultivation.

The essential components of any bioreactor culture system should include a cell culture chamber and the external loop components. The external loop components provide a mechanism for delivery of nutrients to the culture chamber. A peristaltic pump mechanism provides the flow of medium from a fresh medium reservoir to a waste reservoir. For oxygenation, either a bubble oxygenation method or porous membrane with a large surface area is required. A manifold allows escape of waste gases such as CO_2.

Certain factors have been shown to be of importance in the culturing of animal cells which could be addressed with the use of bioreactors.

Adequate medium perfusion is vital for cells to obtain nutrients. There are three methods by which cells in a scaffold may obtain nutrients form the surrounding medium. *Diffusion* is the movement of molecules by virtue of their kinetic energy along a concentration gradient and *perfusion* is the movement of molecules along a hydrostatic pressure gradient. Both methods can readily be utilised in the design of a bioreactor to improve mass transfer. *Osmosis*, which is movement of water from a solution of low concentration of solute to one of a high concentration requires a change in the scaffold material and would thus be an inappropriate option. Bioreactors mixed by mechanical stirring devices have routinely been used to culture mammalian cells, as long the impellers are properly designed and mixing speeds are slow (Thilly & Levine 1979). However, with mechanical stirring, the mixing intensity varies widely and the shear rate is 10-fold higher at the impeller surface than elsewhere in the solution. The resulting shear gradients are associated with nutrient and pH gradients and non-uniform mass transfer rates, which could adversely effect cell growth and function. In addition, the impeller can itself interfere with cell-scaffold interactions in the early stages of implant cultivation. Thus, bioreactors which rely on mechanical mixing may not be optimal for growing tissue-like implants for clinical use (Freed, Vunjak-Novakovic, &

Langer 1993). Bioreactor designs which rely on mixing by secondary fluid flow instead of mechanical mixing have recently been proposed. For example, a uniform microenvironment can be achieved in bioreactors mixed by fluid recirculation. (air-lift reactors, fluidised bed reactors, rotating wall vessels) (Hu & Oberg 1990). These systems improve the mass transfer rates over the course of the culture by increasing the rate of internal fluid recirculation (Posillico 1986).

Shear stress is defined as a force per unit area, acting *parallel* to an infinitesimal surface element and it is primarily caused by friction between particles. Fluids with high viscosity have high shear stress that increase with increasing flow velocity at the shear surface. Cells encapsulated within scaffold systems, unlike cells at the surface, are to some extent protected from shear stresses as they are not directly exposed to the shear surface. In most mammalian culture systems, shear stresses should be minimised because they cause damage to cells and scaffold systems. Goodwin et al cultured hamster kidney cells under various shear stress conditions (Goodwin et al. 1993). Shear stresses ranged from 0.5 x 10^{-3} Nm^{-2} to 0.92 x 10^{-3} Nm^{-2}. They found that glucose utilisation and cellular damage were kept to a minimum at the shear surface using a low shear culture device. In the case of cartilage regeneration, the ability to apply small, controlled shear stresses might help to improve the biochemical properties of the growing implant, since architectural repair of damaged cartilage depends in part on mechanical stresses such as those induced by joint motion (Salter, Simmonds, Malcolm, Rumble, MacMichael, & Clements 1980). Conventional bioreactor systems create a shear stress of 3-10 x 10^{-3} Nm^{-2} (Jessup, Goodwin, & Spaulding 1993). Since cell viability is decreased at shear stresses greater than 5-7 x 10^{-3} Nm^{-2}, most culture systems operate in a high shear stress environment that restricts growth. Bioreactor mixing conditions should thus be optimised to maintain the proper balance between mass transfer rates and shear stresses over the course of chondrocyte proliferation and cartilage regeneration (Freed, Marquis, Nohria, Emmanual, Mikos, & Langer 1993).

Growth of cells under high agitation conditions producing **turbulence** results in a net reduction, which is due both to cell death from altered hydrodynamic forces and to cell loss from the constructs. Growth inhibition may also play a role in the reduction of net growth. In animal cells, glucose uptake is significantly reduced by high levels of agitation. In endothelial cells it has been shown that cell shape, metabolism and pinocytic activity can be strongly affected by fluid flow (Dewey, Jr. et al. 1981). The

results are due, at least in part, to the natural adaptation of endothelial cells to fluid flow in blood vessels. For cell lines other than endothelial, there have been very few studies, however, there are reports of differences in cell metabolism between agitated and stagnant cultures (Bryant 1969;Giard et al. 1979).

The effect of **gravity** should preferably be minimised because it causes sedimentation of substrates and waste products. A culture system which allows a constantly changing gravity vector may allow a more homogenous distribution of matrix components with minimal sedimentation.

The requirement for high cell densities in tissue engineering has been discussed earlier. High cell densities require large culture chambers or medium reservoirs to cope with the high nutritional demands of growing cells. The **volume** required by the cells and their extracellular matrices needs also to be taken into consideration when estimating the volume capacity of a bioreactor. Bioreactors have been developed in various designs and capacities for various biotechnological applications. For example, large bioreactors up to 100,000 L are routinely used for microbial cell production of pharmocological agents such as penicillin. Small bioreactors, with a capacity of up to 0.5 L for tissue engineering are currently at an early developmental stage.

All living human cells require oxygen for their metabolic processes. Conventional methods of **oxygenation** disturb the medium and damage the cells (Cherry & Hulle 1992). Previous designers have suspended particles in horizontally rotating culture chambers which produce a quiescent environment, however, none has been able to provide a means of oxygenation that does not perturb this tranquil environment. Many stirred reactors depend on gas control and diffusion at the gas/medium interface, known as headspace oxygenation, however, these systems are prone to turbulence and bubble formation. Other designs use sparging or airlift techniques, where gas is introduced as minute bubbles and thus lifts the cells and microcarrier constructs up through the medium.

The use of silicone tubing to oxygenate a bioreactor has been previously explored (Fleischaker & Sinsky 1981). In this method a silicone rubber tubing was placed in direct contact with the medium. A gas mixture was circulated through the tubing, passively diffusing into the medium at the coefficient of the particular gas. The authors

found that oxygen transport varied with the rate of agitation and concluded that the problem of oxygenation of large scale cell cultures could be adequately dealt with by membrane oxygenation. Gas diffusion through a silicone membrane could be improved by decreasing the membrane thickness. An example of this technology is extracorporeal oxygenators used in heart-lung machines.

The maintenance of **sterility** is extremely important in cell cultures. Long term cultures require extreme care in handling, especially during changing of medium and specimen collection. Cultures in standard incubators are prone to both fungal and bacterial infections. The warm humid conditions of incubators are ideal breeding grounds for numerous pathogens. Bioreactors should allow easy change of medium and specimen collection to reduce the chance of any infection.

2.6.4 Commercially available Bioreactors for research

Several culture systems are available in the research setting. These include static cell culture, static matrix cultures, roller bottles, stirred suspension culture, airlift bioreactors, and hollow fiber perfused cell systems (Jessup, Goodwin, & Spaulding 1993). Each of these systems has advantages and disadvantages for the analysis of in vitro cell cultures. Static cell cultures are simplest culture systems which provide no shear stress, allow careful morphologic analysis of the interaction of two monolayers of cells, and relatively easy isolation of products secreted into the environment and the retrieval of cells. The disadvantages of monolayers have been discussed previously. Static matrix cultures such as those created in agarose or alginate are very useful for growing cells in a true three dimensional architecture which permits morphologic analysis of cells. However, cell densities in a gel matrix are severely limited by diffusion of nutrients and wastes through the matrix. This limits cell replication and hinders the production of substances secreted into the microenvironment.

2.6.4.1 Roller Bottle cultures

Roller bottle cultures are an excellent method for growing monolayers of cells or cells on microcarriers. High cell densities can be obtained and product isolation is relatively easy. However, the system will exhibit very high shear stresses which may cause cell

damage to cells and constructs. Airlift Bioreactors & Stirred Suspension culture systems allow growth of cells to 10^7-10^8 cells/ml under conditions of fluid mixing. These systems usually use cell coated microcarrier beads. These systems are usually used for production of factors in batch cultures (Dean, Jr. et al. 1987) and not usually used to study the interaction of cells with their matrix. The stirred suspension culture system is a two dimensional system if microcarrier beads are used, but can also be used for three dimensional cultures (Freed, Vunjak-Novakovic, & Langer 1993). Moderate shear stresses are present which result in some cell damage and loss.

2.6.4.2 Hollow fiber-perfusion system

The hollow fiber-perfused sytem is the most successful batch culture system available in the research laboratory. Cells are grown on one side of a semi-permeable membrane while medium is perfused on the other side. This system has no shear stress and allows easy recovery of cells and products for analysis. Three-dimensional growth within the interstices of the fibers is feasible and high cell densities of 10^7-10^8 cells/ml may be obtained. However, this system also suffers from diffusion of nutrients and waste products along the length of the fiber and through the membrane into the thickness of the cell mass.

2.6.4.3 Rotating Wall Vessel (RWV) Bioreactors

The term rotating wall vessel (RWV) comprises a family of vessels, batch fed and perfused which embody the same fluid dynamic operating principles. These principles are

1 Solid body rotation about a horizontal axis which is characterised by
   a) co-location of particles of different sedimentation rates
   b) extremely low fluid shear stress and turbulence and
   c) three-dimensional space freedom

2. Oxygenation by active or passive diffusion to the exclusion of all but dissolved gasses from the reactor chamber, yielding a vessel devoid of gas bubbles and gas/fluid interface, termed zero headspace.

The fluid filled rotating wall vessel (RWV) bioreactor is a recently developed cell culture device that is able to successfully integrate cell-cell and cell-matrix co-localisation and three-dimensional interaction with excellent low-shear mass transfer of nutrients and waste products, without sacrificing one parameter for the other. The RWV bioreactor compares well with the other systems because it combines the favourable aspects of the other systems. It was developed at NASA-Johnson Space Centre and suspends particles by rotating the vessel and spin filter around the horizontal axis. In its simplest form, the RWV has a capacity of 50-500 ml with a spin filter covered with a semipermeable membrane that permits gas diffusion. Non-adherent cells are cultured in suspension, while adherent cells are grown on microcarrier beads. Cells or beads are maintained in suspension by balancing their sedimentation induced by gravity with centrifugation caused by vessel rotation. Rotation of the RWV at speeds of 10-60 rpm maintains particles that are up to 1cm in laminar streamlines, so that individual particles behave as though they were in a continuous fluidised bioreactor. This means that the cells or particles are subjected to a randomised gravity field with low shear stresses. The RWVs provide a suspension culture environment that initially has low shear stress, since particles the size of microcarrier beads between 150 and 175 μm diameter are subjected to stresses of 0.81×10^{-3} Pa. However for larger particles of less than 1cm diameter the shear stresses increase to 0.92×10^{-3} Pa which is still much lower than the shear stresses experienced in conventional systems such as stirred reactors.

Chapter III

Materials & Methods

3.1 Isolation of chondrocytes

Chondrocytes were isolated from slices of bovine articular cartilage using standard protocols previously established at the Institute of Orthopaedics, Stanmore. Metacarpophalangeal joints of young bovines (less than two years of age) obtained from a slaughter house were opened under aseptic conditions within 6 hours of collection. Prior to dissection the hooves were thoroughly cleansed and washed in 70% industrial methylated spirit (IMS) ***(figures 3.1.1 and 3.1.2)***. Cartilage slices were cut from the exposed joint surfaces down to subchondral bone and collected in a 60mm tissue culture dish containing ***(figures 3.1.3 and 3.1.4)*** Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% Foetal Calf Serum (FCS), 40IU penicillin, 2mM L-glutamine, 8mM L-ascorbate and 16 mM Hepes buffer (all from Gibco, Paisley, UK).

The cartilage sections were then diced into approximately $1mm^{-3}$ fine pieces using two sterile scalpels. The medium was then aspirated and the tissue washed in EBSS and transferred to a sterile polypropylene Falcon conical tube ***(figure 3.1.5)***. The cartilage pieces were treated with 10 mls Pronase E (700 IU/ml activity and incubated on a rolamixer for one hour at 37°C) (MERCK 39052 – Streptomyces Griseus ; BDH, Poole, UK) to degrade the non-collagenous matrix molecules and unmask the collagen fibres. To degrade the collagenous matrix, the pronase was decanted and cartilage pieces were digested in 30 mls collagenase (100 IU/ml acivity) (Sigma Type XIa Lot no 45H69995 – Clostridium histolytica ; Sigma, Poole, UK) overnight at 37°C. The enzymes used sequentially are intended to increase permeability to subsequent enzymes by degrading proteoglycans, (Pronase)

The following day, digestate was resuspended with a 10-ml pipette and passed through a 70 μm cell sieve (Marathon Laboratory Supplies) into a fresh Falcon tube to filter out non-digested matter. The tube was centrifuged at 2000 rpm for seven mins, supernatant

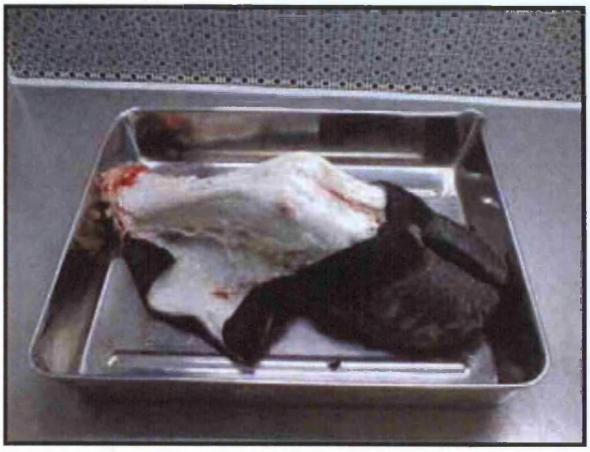


Figure 3.1.1 Dissection of Bovine Hoof

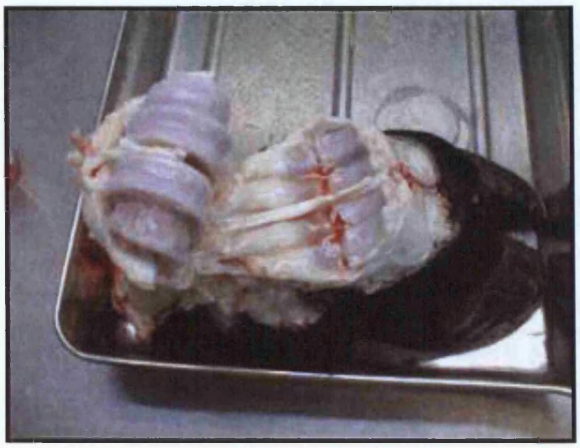


Figure 3.1.2 Dissection of MCP joint

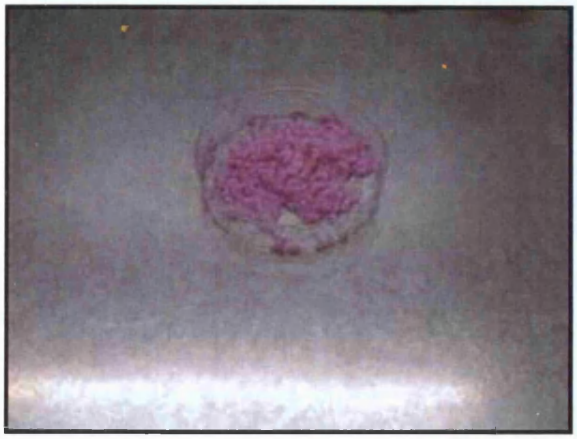


Figure 3.1.3 Cartilage slices in dish

Figure 3.1.4 Cartilage slices in DMEM

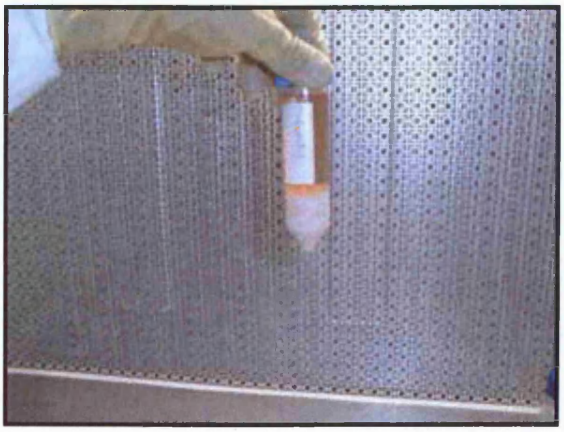


Figure 3.1.5 Tissue washed in EBSS

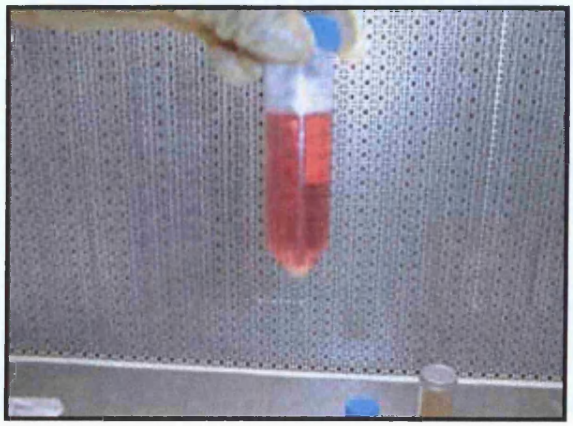


Figure 3.1.6 Cell pellet post-digestion of cartilage resuspended in EBSS

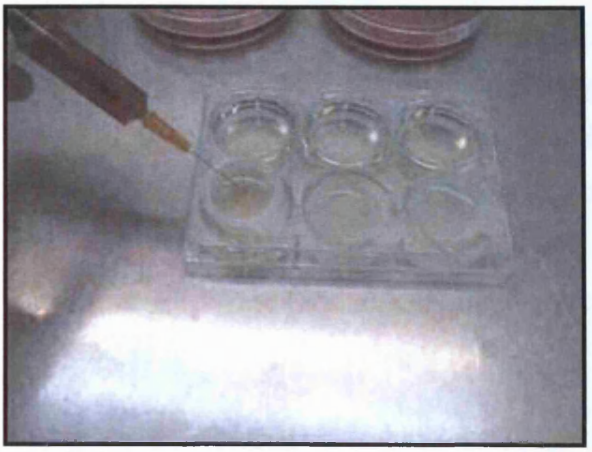


Figure 3.2.1 Alginate/cell suspension dropped into calcium chloride solution

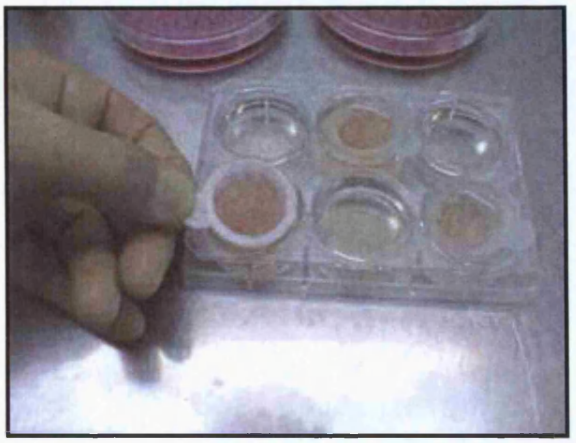


Figure 3.2.2 Alginate/cell beads washed by transferring sieve into fresh medium

Figure 3.2.3 Beads transferred into a 50ml Petri dish

Figure 3.3.1 Beads transferred into vessel and filled with fresh medium through filling port

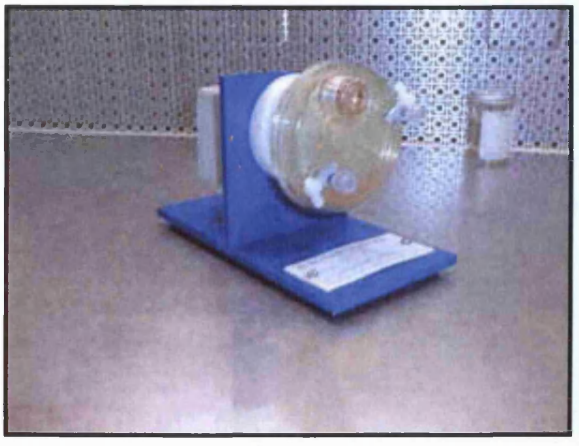


Figure 3.3.2 Rotating Wall Vessel Bioreactor containg alginate beads and medium

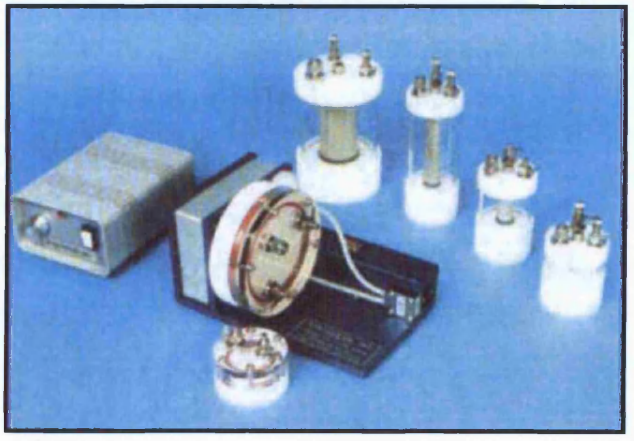


Figure 3.3.3 Synthecon Rotating wall vessel bioreactor with additional components

was removed and the cell pellet gently resuspended in 10mls Earles Balanced Salt Solution (EBSS) (Gibco, Paisley, UK) ***(figure 3.1.6)***. Following two successive washes in this manner, cell pellets from individual hooves were combined and resuspended in 10 mls medium. A 50µl aliquot was extracted and diluted by an equal quantity of 0.4% Trypan blue solution in a microfuge tube and thoroughly mixed. A Neubauer counting chamber was used to perform a cell count using an Olympus light microscope and the trypan blue exclusion technique.

Once the cell concentration was established, the cell supension was centrifuged and resuspended in an approprite quantity of medium to make a final cell density of 20 million cells per ml of solution. The cell suspension containing 20 million cells per ml was utilised in further studies.

When using agarose or alginate solutions a final cell density of 10 million cells per ml of construct was required. The cell suspension was therefore diluted by 50% using the appropriate gel solution.

3.2 Formation of Three dimensional scaffolds

The culture of chondrocytes that have been released from their extracellular matrix is described in this section. The functional aspect of this form of culture is that cell-to-surface attachments are prevented and cells remain spherical. This requirement has been met by various culture systems including alginate and agarose. In order to perform experiments on chondrocytes embedded in these three dimensional scaffolds it was necessary to use previously established techniques at the Institute of Orthopaedics and which are also well documented in previous literature. The methods of construct formation vary according to the material being used. My experiments used collagen, alginate and agarose as scaffold materials as these materials have been particularly successful in chondrocyte cultures. All procedures were performed under strict aseptic conditions using sterile disposable instruments in a flow cabinet to minimise contamination.

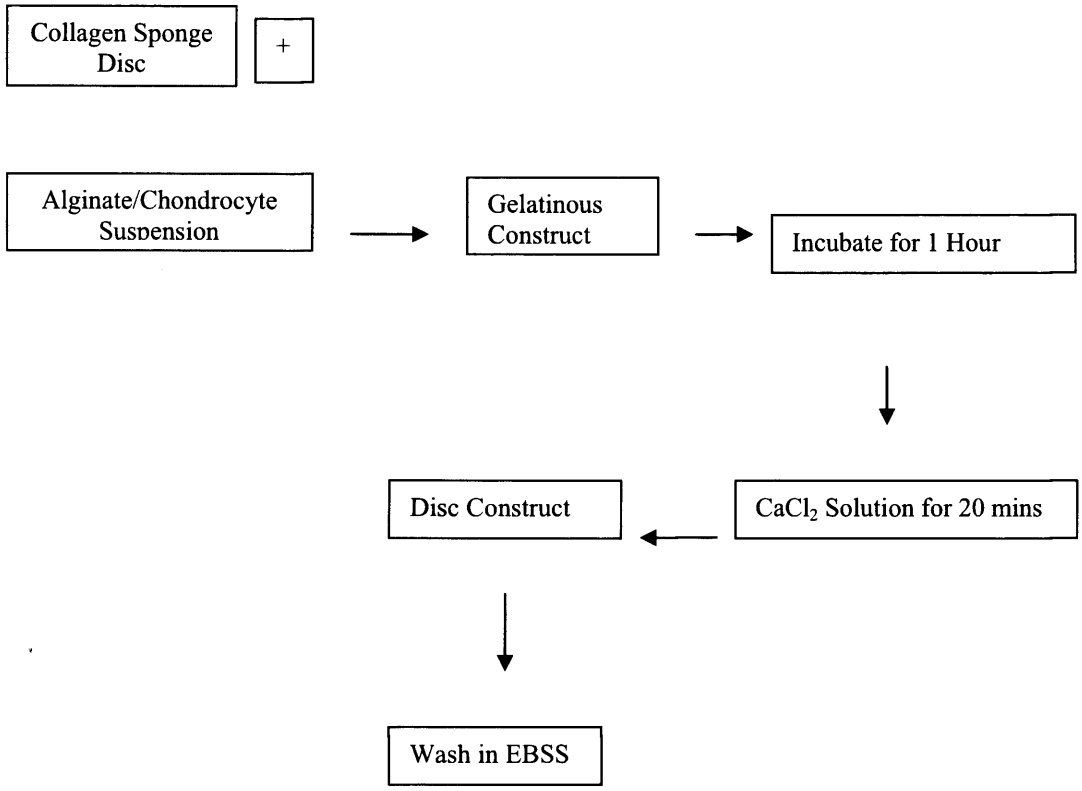
3.2.1 Collagen/Alginate disc formation

Alginate gels are formed by chelation with calcium ions. In order to construct a collagen sponge embedded with alginate gel (containing chondrocytes within) it is vital to fully incorporate the alginate into the interstices of the collagen sponge. As the collagen sponge has a weak architecture which is easily deformed, methods relying on pressure gradients to force or suck alginate into the sponge have led to loss of sponge architecture. The most efficient method of gel incorporation was achieved by simply soaking the sponge in alginate solution. The diagram below outlines the process of construct formation.

A collagen sponge obtained as a sterile flat sheet was aseptically cored out to make discs of 6mm diameter and 4.0mm thickness. The discs were placed individually in a 6 well tissue culture plate. A pre-made 4% Low viscosity Alginate (Kelco) solution in EBSS was autoclaved and added to an equal quantity of cell suspension to make a 2% cell-seeded gel solution of alginate containing a final cell concentration of 10 million cells per millilitre. One hundred millilitre aliquots of this cell suspension was dropped onto each collagen sponge disc. The resulting soft gelatinous constructs were incubated for one hour to allow complete absorption of the alginate into the collagen sponge.

Each construct was then removed and dropped into a well containing 100mmols sterile calcium chloride solution for twenty minutes to allow the alginate to gel. The gelling process resulted in the formation of firm collagen/alginate discs. The discs were then transferred into wells initially containing EBSS followed by wells containing fresh medium in order to wash off the calcium chloride solution.

The collagen/alginate/chondrocyte disc constructs were then ready to be used for further experimental work.



An illustration of the alginate chelating process

3.2.2 Agarose Disc Formation

Low viscosity agarose powder (Type VII, Low temperature gelling, Sigma) was made up to 6% concentration of agarose solution in EBSS and sterilised in an autoclave. Using warm sterile pipettes, the agarose solution was diluted by an equal quantity of cell suspension to form a final 3% agarose/cell solution. Care was taken to prevent the formation of air bubbles. The mixture was then carefully poured into sterile moulds and incubated at 4°C for 20 minutes to allow gelling. The resulting discs were removed from the template and dropped into fresh medium. The discs measured 5mm diameter by 6mm thickness.

3.2.3 Alginate Bead formation

Beads have certain advantages over discs. They are easier to construct requiring minimal handling, they have smaller dimensions and thereby allow improved diffusion and also they are more consistent in their dimensions. The ability of alginate to

instantaneously gel on contact with calcium chloride solution allows it to be used to form small spherical beads. Alginate/cell suspension of 2% final concentration slowly extruded from a 22 gauge needle and dropped into 100 mmol solution of calcium chloride results in the immediate formation of alginate beads (***figure 3.2.1***). From preliminary experiments it was found that on average 85 beads were formed from 1ml of alginate/cell suspension. Care was taken to prevent drops falling from too great a height as this lead to formation of abnormally shaped beads. The best distance for bead formation was found to be 2cm from the surface of the calcium chloride solution. From this height beads were at their most spherical shape and providing the same distance was used and they were expelled at the same rates it was found that all beads were approximately equal in volume and shape.

Using a 6 well plate, 3 wells were filled with 100mmol calcium chloride solution and the remaining 3 wells were filled with fresh medium. A cell strainer was placed in each of the calcium chloride containing wells. Alginate/cell suspension was aspirated into into a 5 ml syringe. A 22 gauge needle was used to slowly expel the solution into the cell sieve. After 5 minutes, to allow complete gelation, the cell sieve was carefully lifted out of the calcium chloride well and placed into the well containing fresh medium. After a further 10 minutes the sieve was moved to the next well containing fresh medium (***figure 3.2.2***). After these two washes the beads were transferred into a tissue culture dish containing fresh medium for further culture (***figures 3.2.3***).

3.2.4 Agarose Bead Formation

Low temperature setting agarose gels immediately when the temperature drops below 4°C. Using the previously described techniques for alginate, it was possible to form agarose beads by dropping agarose/cell solution into fresh medium pre-cooled to a temperature below 4°C. Using a 22 gauge needle, 85 beads on average were formed from 1ml of 3% final agarose/cell solution. The beads were washed twice in fresh medium prior to use in the final culture.

3.3 Cell Culture Methods

3.3.1 Monolayer cultures

Aliquots of the chondrocyte suspension in culture medium (20% FBS in DMEM) were pipetted into 75mm plastic tissue culture dishes to prepare high density monolayer cultures (2 x10^5 cells/cm^2). The cultures were left undisturbed for 24 hrs to allow the cells to attach to the dish, and thereafter they were fed daily with 10ml of fresh culture medium.

3.3.2 Three dimensional scaffold cultures

3.3.2.1 Static tissue culture

The required number of disc or bead constructs were placed in a 50 ml standard tissue culture plate which was filled carefully with 50 mls of fresh warm culture medium. After replacing the lid on the culture dish it was placed within the incubator. Medium change was performed using a pasteur pipette to carefully withdraw the waste medium taking care not to suck up any constructs. Fresh medium was added directly using a pipette. Construct samples were collected using either a forceps or a spoon.

3.3.2.2 Tissue culture in the Rotating cell culture vessel

The Synthecon Inc, Rotating Cell Culture System is a horizontally rotated, bubble free culture vessel with membrane diffusion gas exchange. The culture medium, cells and cell aggregate particles rotate with the vessel and do not collide with the vessel walls or other damaging objects. Destructive shear forces are minimised because this system has no impellers, air lifts, bubbles, or agitators. Gel constructs establish a uniform, very low shear, fluid suspension orbit within the horizontally rotating culture vessels. As the constructs grow or change in weight, the rotation speed is adjusted to compensate for increased sedimentation rates.

3.3.2.3 Experimental Protocol

The vessel was transferred to a sterile hood. The end caps were removed and placed on sterile alcohol pads or in sterile petri plates. The disc constructs were placed into the vessel through the ½ inch port.

25 disc constructs and 200 beads were used as a maximum filling capacity as it was found that a greater number than this resulted in undesired frequent collisions between constructs. The ½ inch port cap was applied firmly. Using a 50ml syringe the vessel was filled slowly through one of the syringe ports. The opposite syringe port was left open so as to allow escape of air as the syringe chamber was filled with medium. During filling, the vessel was held such that the open port was above the filling port. This ensured that all constructs remained at the bottom of the chamber and did not obstruct the outlet. It also ensured that air bubbles were taken to the top outlet port for evacuation from the vessel.

After filling the vessel completely it was laid flat on a sterile surface. Tapping lightly on the sides and slowly injecting more medium into the vessel resulted in escape of any remaining small bubbles from the open port. The ports were then both locked and the syringe removed. Both ports were then wiped.with alcohol and sealed with caps. The final steps were repeated if any remaining bubbles were discovered.

The vessel was wiped with alcohol to remove any remnants of medium or contamination from its external surface. It was then attached securely to the rotator base in a humidified CO^2 incubator. The initial rotation speed was set to 6 to 8 rpm and was adjusted to higher speeds as necessary. With the speed properly adjusted, the constructs formed a fluid orbit within the vessel and did not collide with each other or the edges of the vessel.

3.3.2.4 Media change

The power was switched off, the vessel immediately removed from the base and taken to a sterile biological hood. The end caps were removed and placed on sterile alcohol pads. The constructs were allowed to settle at the bottom with the ½ inch port and valves rotated up. A 50 ml syringe full of fresh culture medium was attached to one

syringe port (*figure 3.3.1*). An empty 50 ml syringe was attached to the second port in order to collect the waste medium. The valves were opened and fresh warm medium was slowly injected into the vessel while at the same time the empty syringe was used to withdraw waste medium. During this procedure the vessel was placed base down with ports up to expel air bubbles from under the ports. Occasionally some manoeuvring was required to ensure that all air bubbles were expelled. When all the medium had been replaced, the syringe valves were shut and syringes discarded. Waste medium was collected for analysis if required. The ports were wiped with alcohol and caps replaced. The vessel was reattached to the rotator base and power switched on (*figures 3.3.2 and 3.2.3*).

3.3.2.5 Sample collection

Under sterile conditions with the vessel placed base down the ½ inch port cap was removed by unscrewing and was placed in a sterile petri plate. In the case of disc constructs, sample discs were removed using a fine forceps from the vessel and collected in a sterile petri plate, medium sampling was performed using a 2ml syringe to collect medium directly from the vessel via the ½ inch port. Care was taken at all times to prevent sucking up disc constructs into the syringe as this inevitably lead to disc damage and fragmentation. The edges of the port was cleansed with alcohol and the cap screwed back into place. The culture medium was then replaced using the two 50 ml syringe technique previously described. If using beads, the method of sampling differed as it was not possible to collect beads directly from the vessel using forceps because the beads were very slippery. The complete contents (including beads) of the vessel were emptied into a large sterile petri dish and the beads collected using a small scoop or spoon. Medium for sampling was collected using a 2ml syringe. A pasteur pipette was then used to remove the remaining waste medium. The beads were reinserted into the vessel via the ½ inch port and fresh medium was added to the chamber using the previously described technique.

3.4 Methods of Analysis

3.4.1 Biochemical

Chondrocyte/alginate gels were disrupted and digested by addition of a solution (Papain Digest Buffer) containing 55mM sodium citrate (BDH), 150 mM sodium chloride (BDH), 5mM cysteine hydrochloride (Sigma), 5mM EDTA (BDH) and 0.56 units.ml^{-1} papain (Sigma) into a microfuge tube. The samples were incubated at 60°C for 24 hours. Following incubation the digested samples were stirred on a vortex mixer.

Chondrocyte/agarose gels were disrupted and digested by addition of a solution containing 55mM sodium citrate (BDH), 150 mM sodium chloride (BDH), 5mM cysteine hydrochloride (Sigma), 5mM EDTA (BDH) into a microfuge tubes. They were incubated at 70°C for 30 mins until molten and then cooled to 40°C for addition of 0.56 units.ml^{-1} papain (Sigma) and 10 units.ml^{-1} agarase (Sigma). After overnight incubation at 37°C followed by vortex mixing they were ready for analysis

3.4.1.1 DNA assay (Hoechst 33258)

The techniques described by Rao et al (1992) and Rago et al (1990) were used.

Calf thymus DNA (1mg.ml^{-1}) was used for preparation of the standard curve. Serial 1:2 dilutions of the 20μg.ml^{-1} standard in a 1:1 solution of saline sodium citrate (containing 0.4% SDS) and Papain digest buffer were performed to produce dilutions ranging from 20μg.ml^{-1} to 0.31μg.ml^{-1}. 100μl of each standard was then pipetted into the appropriate wells in a fluorimetric well plate.

One hundred microlitres Hoechst 33258 (1μg.ml^{-1}) was added to each well plate and after gentle shaking, the plates were read on a fluorimeter with 355nm excitation filter and emission at 460nm.

4.1.2 Glycosaminoglycan Assay

Quantification of total sulphated glycosaminoglycans (GAG) in chondrocyte cultures was necessary for the complete assessment of the metabolic profile of the system and was performed using the 1,9-dimethylmethylene blue (DMB) dye method at pH 3.0 (Farndale et al 1986). The DMB assay relies on spectrophotometric detection of a metachromatic shift in the absorbance maximum from 600nm to 535nm in DMB which occurs when the cationic dye binds to sulphate and carboxyl groups present in GAG chains.

For agarose, the assay is relatively simple. Forty microlitres of papain digested sample was added to 250µl of DMB (pH 3.0) in wells of an absorbance plate. Absorbances were determined using a Chondoitin-6-Sulphate (Sigma) standard curve (0-100µg.ml^{-1}) from 0µg.ml^{-1} to 50µg.ml^{-1}.

Measurement of total sulphated glycosaminoglycans in cell culture digests containing alginate using DMB (pH 3.0), is complicated by the fact that carboxyl groups of alginate bind with DMB leading to the formation of complexes which interfere with the spectrophotometric detection of sulphated GAG-DMB. DMB at pH 1.5 has been shown by Enobakhare et al (1996) to be the most accurate pH level for sulphated GAG assays in alginate systems. Forty microlitres of papain digested sample was added to 250µl of DMB (pH 1.5) in wells of an absorbance plate. Absorbances were determined using a chondoitin-6-sulphate standard curve (0-100µg.ml^{-1}).

3.4.2 Assessment of Cell viability

A Live & Dead Cell Staining Kit (Sigma) was used to provide a two-colour fluorescence staining on live cells (green) and dead cells (Red) using two probes. Calcein AM stains live cells green while Ethidum Homodimer stains dead cells red. These probes measure two recognized parameters of cell viability — intracellular esterase activity and plasma membrane integrity. The assay principles are general and applicable to most eukaryotic cell types, including adherent cells and certain tissues, but not to bacteria or yeast.

3.4.3 Histological Analysis

Specimens collected for histological analysis require fixation. The effect of a fixation is to prevent autolysis and putrefaction of the tissues and to render them resistant to damage by osmotic pressure. These changes may be retarded by low temperature or by the use of a chemical fixative. Providing the specimen is fixed as soon as possible, fixatives also help to preserve the specimen in a lifelike form. The type of fixative used depends on the nature of analysis ie standard histological staining, electron microscopy or immunohistology. Fixatives differ in their pH, type of buffer and osmotic strength. Animal tissues have a pH around neutrality, and the best results are usually given by fixatives with a pH range 7.0 to 7.4. Buffers are required to set the pH, and maintain it at the desired value, despite the effects of the reaction of the fixative with the tissue. The two most commonly used buffers are Cacodylate and Phosphate. As phosphate seemd to have a destructive effect on alginate, only cacodylate buffer was used in this project. It is recommended to use a fixative having approximately 60% of the osmotic pressure of the tissue fluids and to use a quantity of about 50-100 times the volume of the tissue. Buffers usually vary around 0.05 to 0.2 Molar and the final concentration is dictated by the consideration of the compromise between osmotic strength and buffering capacity.

3.4.3.1 Preparation of fixatives

The three fixatives used in this research project were osmium tetroxide, glutaraldehyde and formaldehyde. The properties of these three fixatives are summarised in the table below.

	Osmium Tetroxide	Glutaraldehyde	Formaldehyde
Fixes proteins	Yes	Yes	Yes
Lipids	Yes	No	Yes
Carbohydrates	No	Yes	No
Imparts contrast	Yes	No	No
Penetration	Slow	Fast	Very fast
Reaction with tissue	Very slow	Fast	Slow

Semi-permeability destroyed ?	Yes	No	No
Expensive ?	Yes	No	No

The fixative used for standard histological staining and immunohistological techniques was formaldehyde. It fixes most cartilage components with minimal reaction with the tissue itself and therefore specimens could all be analysed at the end of an experiment with good preservation of original structure. The fixative was prepared in sodium cacodylate (NaCaCo) buffer (0.1 M NaCaCo pH 7.4 in 2% paraformaldehyde).

The fixative used for electron microscopy was glutaraldehyde followed by osmium tetroxide. Glutaraldehyde gives excellent preservation of fine structure but begins to react with the tissue and forms cross-links between molecules in the specimen. Mg^{++} is added to prevent artefacts due to the lipid component, which it does not fix. Its poor penetration makes it suitable only to be used for bead constructs. The fixative was prepared in NaCaCo buffer (0.1M NaCaCo pH 7.4 in 1.5% glutaraldehyde). Osmium tetroxide was the first fixative used for electron microscopy, it fixes proteins and lipids, but not carbohydrates and due to its heavy density, it imparts electron contrast. It was used in the preparatory stages of electron microscopy.
Refer to appendix for preparation of buffer solutions.

3.4.3.2 Processing of specimens

Dehydration

The fixative solutions are not miscible with paraffin wax, therefore preliminary dehydration with alcohol was necessary. The specimen was passed through a series of more concentrated alcohol baths. The times that were used are summarised in the table below.

Container	Fluid	Time
1	10% formalin	overnight
2	70% alcohol	2 hours
3	90% alcohol	2 hours
4	absolute alcohol	1 ½ hours
5	absolute alcohol	3 ½ hrs
6	absolute alcohol	1 ½ hr

When processing beads it was useful to wrap them up in tissue paper to prevent them being washed away.

Clearing

'Clearing' or 'de-alcoholisation' is the term applied to removal of alcohol from the specimens. Clearing agents should be miscible with both alcohol and paraffin wax. The agent used in was Xylene. It is the the most rapid of clearing agents but may cause shrinkage and hardening of the tissue.

Impregnation with paraffin wax

The tissues were thoroughly impregnated with wax by carrying them from the clearing agent through three or more baths of molten paraffin wax in the embedding oven. For both bead and disc constructs the impregnation time in the molten wax was 3 hours with three changes of wax.

Moulds for embedding

The Leuckhart embedding boxes were used for the majority of the work. Using Institute of orthopaedics protocols, the specimens were embedded in wax and were ready for sectioning. In the wax state, specimens can be stored for very long periods and may be retrieved for future studies

Section cutting

The microscope is designed to facilitate the study of animal tissue by transmitted light and for this purpose the tissue must be sliced into thin sections. These were cut at a thickness of 8 microns using a sledge microtome which ensured uniform sectioning.

Mounting sections on slides

Several glass slides were cleaned and specimen code inscribed on the frosted surface using a pencil. Adherent slides were used for specimens requiring immunohistological analysis. Sections were mounted onto the slides using a warm

water bath to help unravel the creases. The slides were then placed on a hot plate to dry before staining was commenced.

3.4.3.3 Biological staining

Biological material is stained in order to render the different constituents of tissues and cells more readily visible. Various cellular elements may have different refractive indices, which allows partial identification of unstained material. For detailed study, however, staining is preferred.

After the sections were stained they were prepared as permanent preparations for microscopic examination. This was accomplished by mounting the sections in a synthetic mounting medium (DPX – Dibutylphthalate + Xylene).

Haematoxylin and eosin

Haematoxylin is a natural dye derived by ether extraction from the wood of a Mexican tree. On its own it has poor staining properties and is therefore normally used in conjunction with a mordant such as alum. This enables demonstration of the **nucleus** as a light transparent blue or red depending on the acidity of the counterstain. When counterstained with an acidic dye such as eosin it produces a faint blue. Iron, another mordant, causes a more intense grey-black staining which becomes a light blue on acid counterstain. Alum haematoxylin (ie Mayer's, Ehrlich's and Harris's) was used in conjunction with eosin for demonstrating the general structure of our specimens. Iron haematoxylin (ie Weigart's) was used only when it was necessary to specifically demonstrate cell nuclei.

Technique

1. Rewax, hydrate in descending alcohol concentrations
2. Stain in Ehrlich's haematoxylin (20 mins)
3. Wash briefly in running tap water
4. Differentiate in acid alcohol (2-3 dips)
5. Wash in running tap water until blue (5 mins at least)
6. Counterstain in 1% Eosin (2 mins)

7. Differentiate in running tap water until desired colour is obtained
8. Dehydrate, clear and mount in DPX

Safronin-O

Safranin-O is a cationic dye consisting of a mixture of diaminophenyl-ditolazonium chloride and diamino-o-tolyditolazonium chloride, it can vary in shades from blue, red, orange to yellow depending on the batch used. It is used for the demonstration of **chondroitin sulphate** and **keratan sulphate** in the matrix of cartilage. It does not bind to collagen and therfore distinguishes cartilage matrix from ligament, tendon and bone (Rosenberg 1971). When used as a counterstain with haematoxylin there is an intense staining of the matrix around condrocytes. It was originally described to be used as a 0.1% solution for cartilage, however we found it to produce intense staining after 2 minutes.

Technique

1. Rewax, hydrate in descending alcohol concentrations
2. Stain in Mayers haemalum (15 mins)
3. Wash briefly in running tap water
4. Differentiate in acid alcohol (2-3 dips)
5. Wash in running tap water until blue (% mins)
6. Counterstain in 0.1% Safranin-O (2 mins)
7. Wash quickly in running tap water
8. Commence dehydration in 95% alcohol, clear and mount in DPX

Other stains

Picrosirius staining, a strong anionic dye, stains collagen by reacting, via its sulphonic acid groups, with basic groups present in the collagen molecule. The elongated dye molecules are attached to the collagen fibre in such a way that their long axes are parallel. This parallel relationship between dye and collagen results in enhanced birefringency. However sections fixed in paraformaldehyde or especially glutaraldehyde may show a reduction in the amount of dye bound due to the cross links formed at the amino groups in collagen .

Alcian blue, a cationic dye, was established for proteoglycan demonstration in 1965. It differs from other dyes in that it does not undergo metachromatic change. It stains much slower than other cationic dyes which can sometimes be a problem as cartilage tissue has a tendency to detach itself from the slide when left in aqueous solution for long periods.

3.4.4 Immunolocalisation

The streptavidin biotin-immunoperoxidase method was used for the immunolocalisation of **collagen** (types I, II IX and X). This process was also used for detection of Ki-67 nuclear antigen. This is a nuclear antigen found only in **proliferating cells** and gives a reliable indication of the presence of healthy replicating chondrocytes.

The Streptavidin-biotin-peroxidase method relies on using 3,3 –Diaminobenzidine (DAB) as a precipitating substrate for the detection of peroxidae-antibody-antigen complexes.

3.4.4.1 Technique

Sections were taken through 70% alcohol. A blocking step using 200µl hydrogen peroxide:12ml methanol was required to block activity of endogenous peroxidase. As the specimens were fixed in paraformaldehyde, it was necessary to apply pre-treatment to unmask the antigenic sites. For alginate specimens the peroxidase blocked sections were pre-treated with trypsin solution (0.1% trypsin (Sigma), 0.1% $CaCl_2$ in distilled H_2O) at 37°C for 10 minutes. For localisation of Ki-67 (proliferating nuclear antigen) the trypsin pre-treatment was not successful and therefore heat treatment was used. Slides were placed in a container and covered with 10mM citrate buffer, pH 6.0 containing 0.01% EDTA. Microwaved for 5 minutes and buffer changed to fresh buffer. After a further 5 minutes in the microwave, the buffer was removed and specimens washed in PBS.

After enzyme pre-treatment the sections were washed in tap water for 10 minutes and soaked in Tris Buffered Saline (TBS) (0.05M TRIS, 0.15M NaCl, pH 7.6) for 10 minutes. To suppress non-specific binding of IgG, the sections were blocked with

normal rabbit serum (Dako A/S, Denmark) 1:5 in TBS for 20 mins. Approximately 100µml of reagent was used per slide and all steps were carried out with caution to prevent drying of the sections.

Sections were then incubated with primary antibody for 30 minutes at room temperature.

Primary antibody details

Antigen	1°Antibody	Dilution	Source	Control
Type I Collagen	Col-I	1:100	Sigma	Bovine tendon
Type II Collagen	CIICI	1:2	DSHB	Bovine cartilage
Type IX Collagen	B3-1 and D1-9	1:1 with D1-9	DSHB	Bovine cartilage
Type X Collagen	MC7	1:100	donated	Bovine cartilage
Nuclear antigen	Ki-67	1:50	donated	Tonsil

DSHB = Developmental Studies Hybridoma Bank (University of Iowa, USA)
DR ALVIN P.L KWAN donated type X Collagen (Dept of Biochemistry, Cardiff University)

The primary antibody was then rinsed with TBS for 5 minutes and biotinylated rabbit anti-mouse secondary antibody was applied. The sections were then incubated at room temperature for 30 minutes before being rinsed with TBS fro 5 minutes. Strept Avidin-Biotin complex (Dako K0377, Denmark) was applied for 30 minutes. The DAB solution ($0.7mg.ml^{-1}$ DAB, $0.7mg.ml^{-1}$ urea hydrogen peroxide, Tris buffer 0.06M, Sigma) was prepared.

The sections were washed in tap water and counterstained with Harris's haematoxylin for 2 minutes. After a 5 minute wash in tap water and differentiation in acid alcohol they were dehydrated, cleared in xylene and mounted in DPX.

3.4.5 Specimen preparation for Immunolocalisation

3.4.5.1 Specimen preparation

Each specimen was primarily fixed in Glutaraldehyde and then washed in buffer. Secondary Fixation with Osmium tetroxide was performed followed by a further buffer Wash. Specimens were then organically dehydrated and this was followed by resin infiltration. After blocking (Resin cured at 70°C), they were sectioned onto grids using

an ultramicrotome. Stained with Heavy Metals (+/- Rotary evaporation) and finally ready for the transmission electron microscope.

3.4.5.2 Embedding and sectioning

The specimens were embedded epoxy resin as it has a high melting point to withstand the electron beam and this is then polymerised to harden the block. Polymerisation was performed at 60°C for 24 hours. The solid blocks were trimmed and sections from the solid blocks were cut at 50nm thickness using an ultramicrotome. After staining with a heavy metal i.e. lead, osmium tetroxide or Uranium salts, the sections were analysed under the TEM and representative photographs were taken.

3.4.5.3 Control specimens

Figures 3.4.1, 3.4.2 and 3.4.3 are control specimens displaying the appearance of a normal section of bovine articular cartilage. *Figure 3.4.1* is a Safranin-O stain. There is intense staining of proteoglycans in the extracellular matrix. There is significant staining throughout the whole specimen. *Figure 3.4.2* is a haematoxylin and eosin stain. The appearance of cells and cell clusters can be identified. The spindle configuration of the chondrons is well displayed. On occassions there are clusters composed of 4 or more cells with surrounding matrix. *Figure 3.4.3* is a collagen type II immunolabel. Collagen type II is detected throughout the cartilage section and greater amounts appear to occur towards the periphery. Collagen type I immunolabelling was also performed. There were no identifiable levels of this detected in the normal articular cartilage.

Figures 3.4.4 and 3.4.5 are control specimens of bovine Achilles tendon. The tissue has been immunolabelled with collagen type I. There is positive staining throughout the tendon, in contrast to articular cartilage, which does not stain for collagen type I. The distribution of tendon fibres and their longitudinal arrangement is well demonstrated and the presence of considerable quantities of collagen type I is confirmed. There was no positive collagen type I staining in articular cartilage specimens.

Figure 3.4.6 is a control specimen of bovine tonsil tissue. The immunolabel is Ki-67 which localises nuclear antigen from dividing cells. This immunolabel is specific for dividing cells and demonstrates the region of high cell nuclear activity in tonsil tissue.

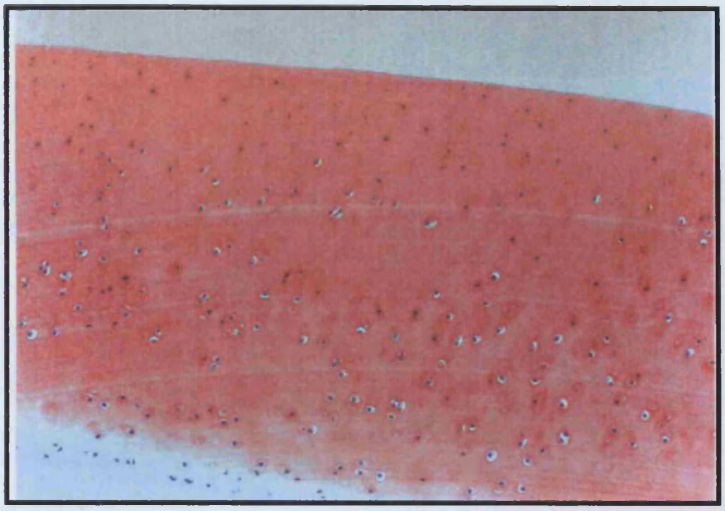


Figure 3.4.1
Safranin-O. Magn X4.
Human Articular Cartilage control.

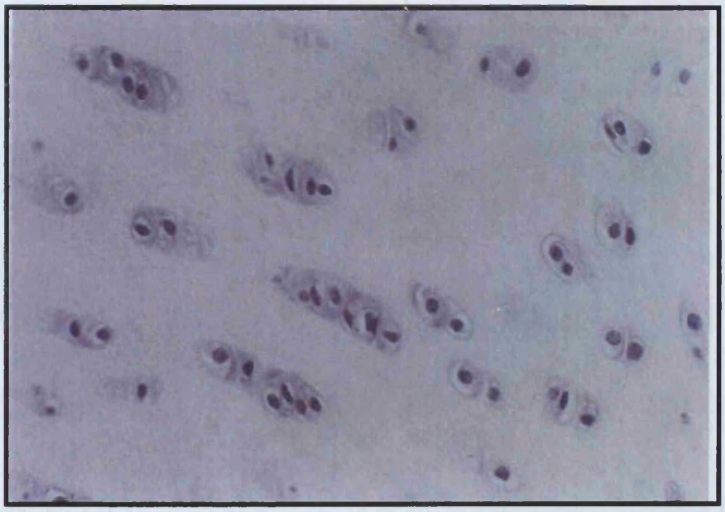


Figure 3.4.2
H&E Stain. Magn X20.
Human Articular Cartilage control.

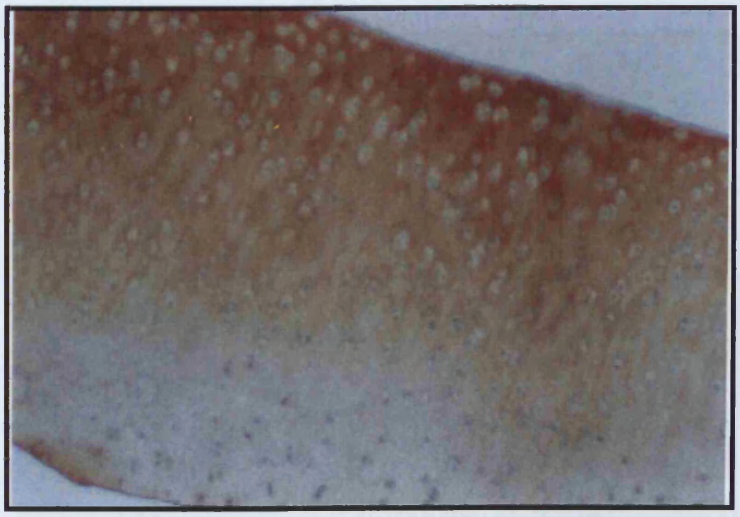


Figure 3.4.3
Collagen Type II Immunostain. Magn X4.
Human Articular Cartilage control.

Figure 3.4.4
Collagen Type I
Immunostain. Magn X20.
Bovine Achilles Tendon
control.

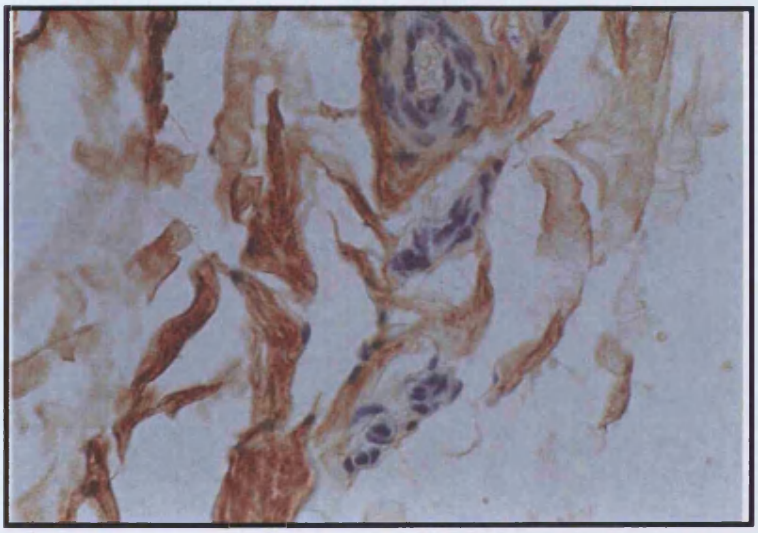


Figure 3.4.5
Collagen Type I
Immunostain. Magn X40.
Bovine Achilles Tendon
control.

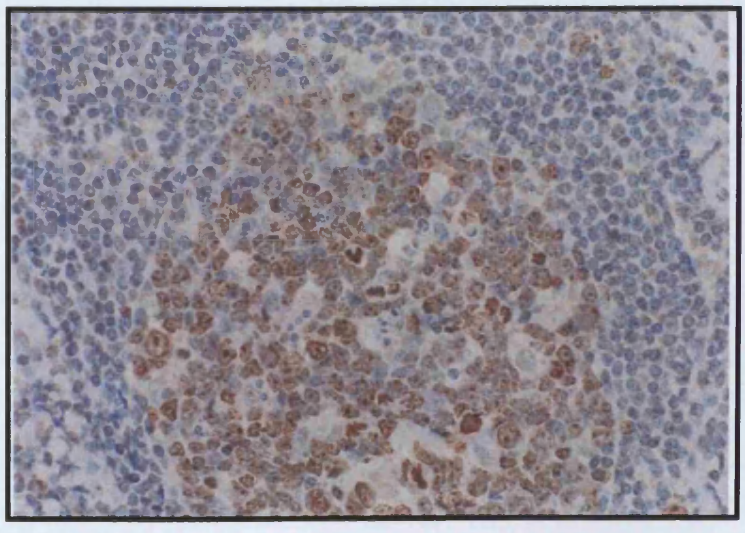


Figure 3.4.6
Ki67 Immunostain.
Magn X4.
Human Tonsil tissue
control.

Only the dividing cells are positively stained whereas the cells with lower mitotic activity do not stain.

3.4.6 Statistical Methods

Statistical comparison between biochemical data obtained from static and RWV culture results was performed using unpaired students t-tests. Values of $p<0.05$ were set to represent statistically significant differences. The Statistical Software for Social Sciences (SPSS) was used for statistical analysis.

Chapter IV

Results

Collagen/Alginate Constructs

Experiments to investigate the in-vitro use of collagen/alginate constructs for chondrocyte culture

4.1 Effect of roller bottles on collagen/alginate constructs

4.1.1 Protocol

The aim of this experiment was to assess the architectural integrity of the collagen/alginate constructs and assess the distribution of chondrocytes within the constructs over a period of 16 days in culture. The culture systems being compared were a static system versus a dynamic system.

Thirty five collagen/alginate disc constructs containing chondrocytes were formed using techniques described in the Materials and Methods section. Five constructs were fixed in paraformaldehyde immediately after formation. Fifteen constructs were cultured in roller bottles on a rolamixer rotating at 20 revolutions per minute and the remaining 15 in roller bottles held statically throughout the culture period. All cultures were incubated in a 37°C incubator in air. Five specimens were placed in each roller bottle with 20mls of culture medium in each bottle. The culture medium was replaced with fresh medium at 48 hr intervals. Five specimens were collected from each culture system at time points of 5 days, 10 days and 14 days. After collection, All specimens were immediately fixed in paraformaldehyde for histological analysis.

Standard haematoxylin and eosin staining as described in the methods was performed on all samples in a batch at the end of the experiment.

4.1.2 Results

Naked eye observation revealed mild disintegration of the constructs after day five in culture. White clumps of construct debris material settled at the bottom of the roller bottles and was later histologically confirmed to be clumps of fragmented alginate. By day 10 there was moderate to severe disintegration of the constructs and there was a considerable loss of alginate from the construct meshwork. This resulted in mechanical weakness of the constructs and the process was visible in both the rolamixer and static cultures. The loss of alginate was more pronounced in the roller bottles cultured on a rolamixer.

Day 0

Figure 4.1.1 shows the collagen sponge at day zero time point. Alginate is more concentrated around the outer edges of the collagen sponge rather than in the central regions. The alginate stained heavily in certain areas were there were greater quantities. The collagen sponge architecture appeared compact and relatively well organised. Figure 4.1.2 taken at higher magnification reveals that the chondrocyte distribution was good with cells present within the construct.

Day 5

Figure 4.1.3 and *figure 4.1.4* shows a typical specimen at day five in static culture. The noticeable features are that there was an overall reduced amount of alginate present within the collagen sponge as compared to day zero. There was relatively more alginate around the edges of the collagen sponge compared to central regions. Central regions of the sponge were becoming partly devoid of alginate. The higher magnification photograph in figure 4.1.4 confirms the above findings and also shows a reduction in overall chondrocyte numbers.

Figures 4.1.5 and 4.1.6 show a typical specimen at day five in a dynamic rolamixer culture. The loss of alginate from within the constructs appeared more marked than the static cultures. Chondrocytes were also fewer in concentration. The most marked feature

however was the loss of construct architecture which occurs particularly around the margins.

Day 10

Figures 4.1.7 and 4.1.8 show a typical specimen at day 10 in a static culture. The findings were quite similar to day five in that there was a gradual loss of alginate from the collagen sponge associated with a deficiency in chondrocyte numbers. At higher magnification, disruption in the collagen sponge architecture was beginning to occur despite the culture being in static conditions.

Figures 4.1.9 and 4.1.10 show a typical specimen at day 10 in a rolamixer culture. The collagen sponge architecture was clearly becoming abnormal at this stage with collagen fibres losing their meshwork characteristic and becoming detached from each other. Small amounts of alginate however was visible within the collagen sponge and chondrocytes were even visible within the sponge embedded within portions of alginate.

Day 14

Figures 4.1.11 and 4.1.12 show a typical specimen at day 14 in a static culture. The collagen meshwork was now considerably disrupted and the collagen fibres were spread out and were becoming fragmented. Some areas retained alginate together with cells which on higher magnification were seen to have divided with small regions of surrounding matrix.

Figures 4.1.13 and 4.1.14 show a typical specimen at day 14 cultured on a rolamixer. In contrast to the static device there was complete disruption of the collagen sponge with marked fragmentation of the collagen fibres. Alginate pieces were identified completely dissociated from the collagen fibres. Chondrocytes were not visible at this stage.

4.1.3 Conclusion

The collagen sponge incorporated the alginate well on initial formation of the construct and the chondrocytes were well distributed within the construct. However, it appears that with increasing lengths of culture time, the collagen sponge became disrupted, most

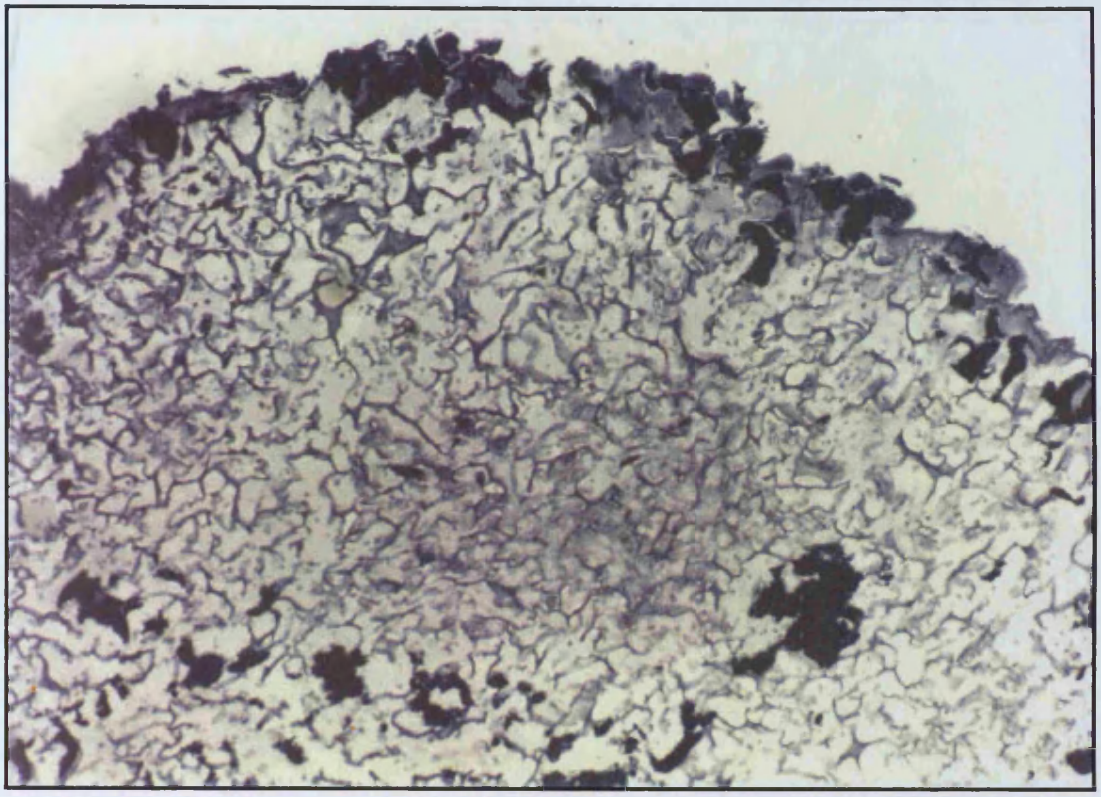


Figure 4.1.1 Magnification x4. H&E Stain.
Collagen/Alginate disc construct taken at day Zero

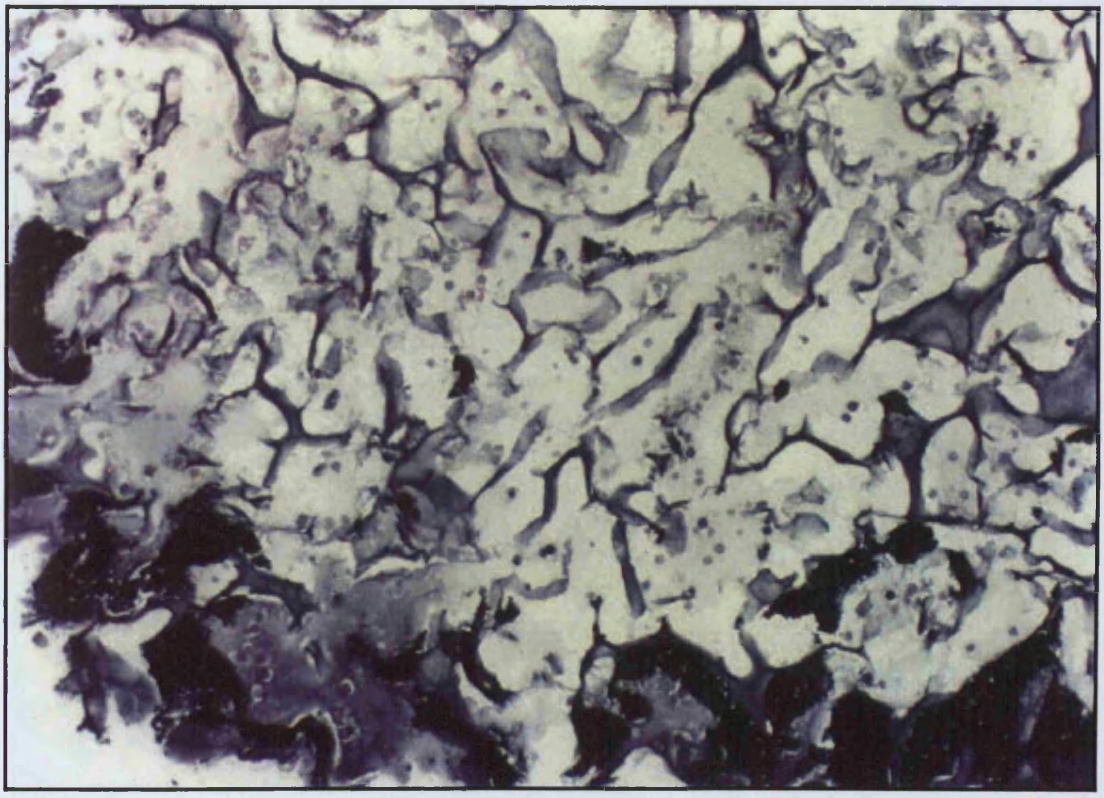


Figure 4.1.2 Magnification x10. H&E Stain.
Collagen/Alginate disc construct taken at day zero

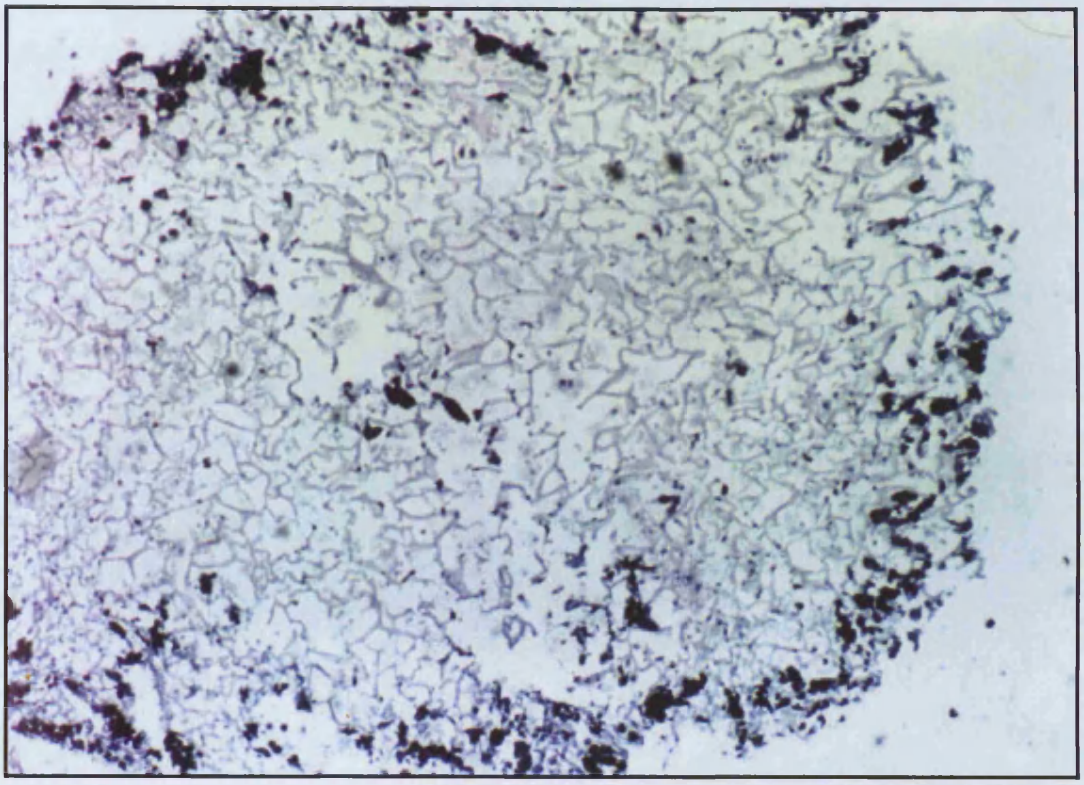


Figure 4.1.3 Magnification x4. H&E Stain.
Collagen/Alginate disc construct taken at day 5. STATIC.

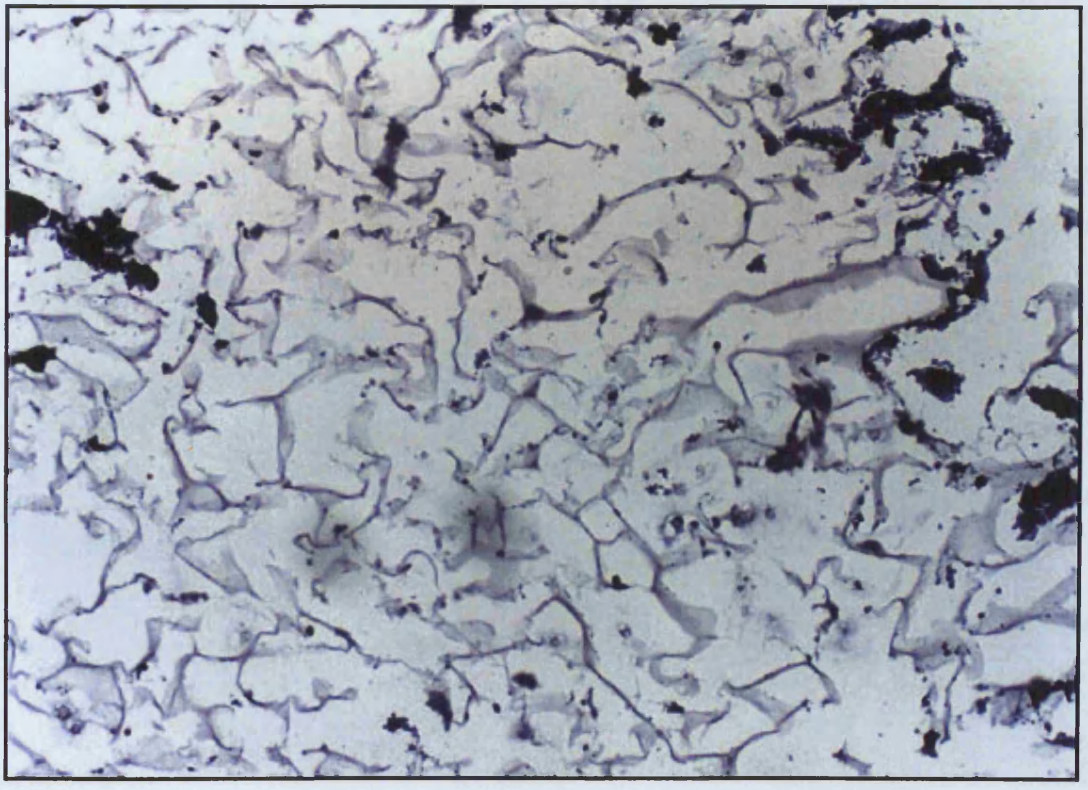


Figure 4.1.4 Magnification x10. H&E Stain.
Collagen/Alginate disc construct taken at day 5. STATIC

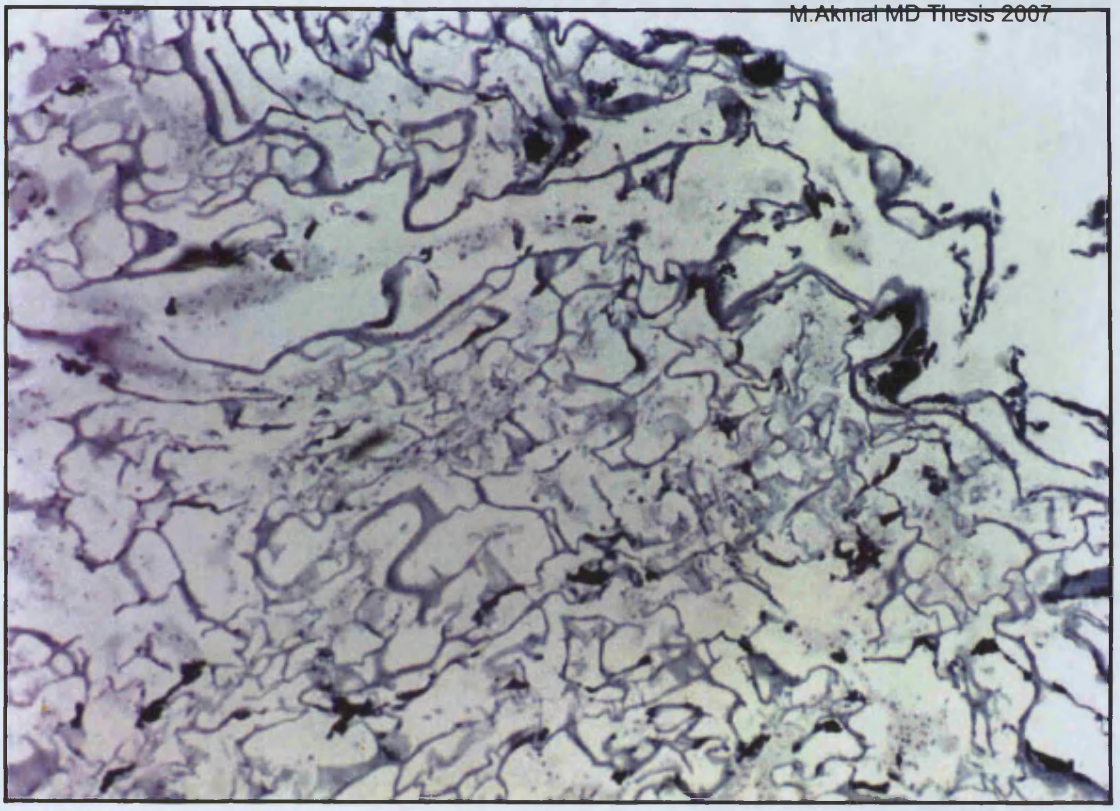


Figure 4.1.5 Magnification x4. H&E Stain.
Collagen/Alginate disc construct at day 5. ROLAMIXER

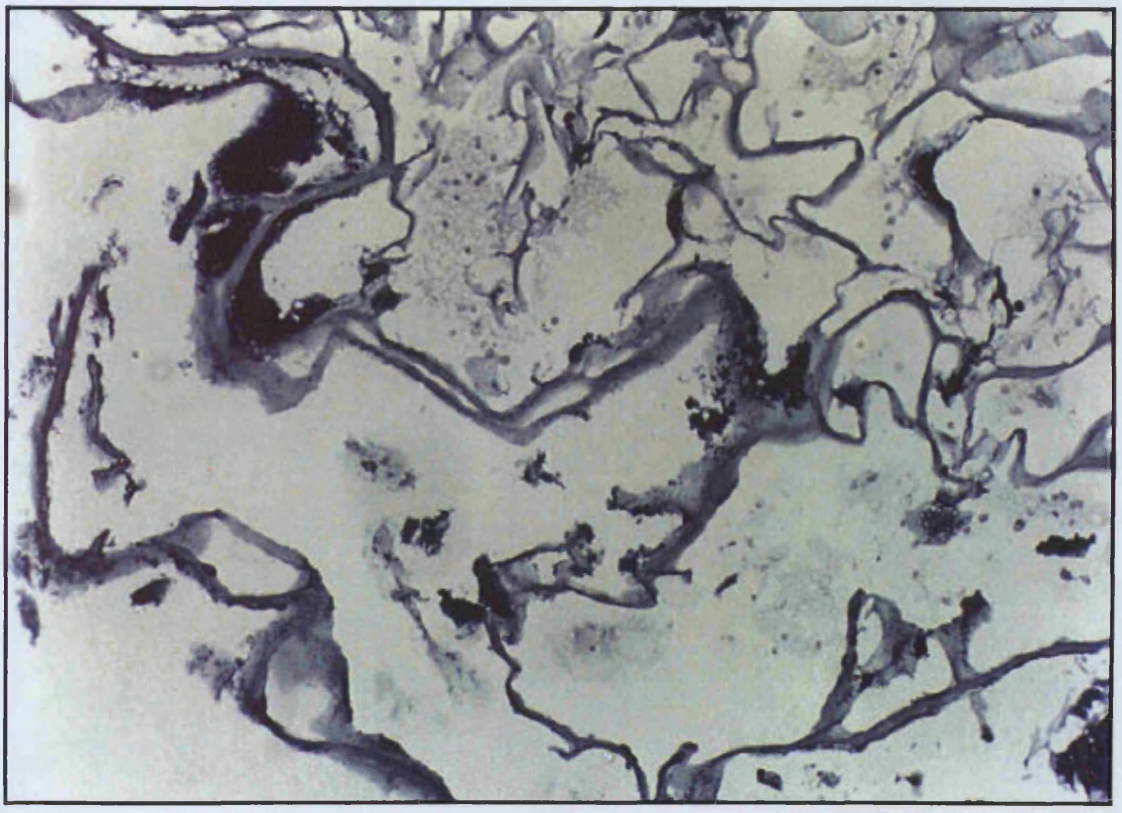


Figure 4.1.6 Magnification x10. H&E Stain.
Collagen/Alginate disc construct at day 5. ROLAMIXER

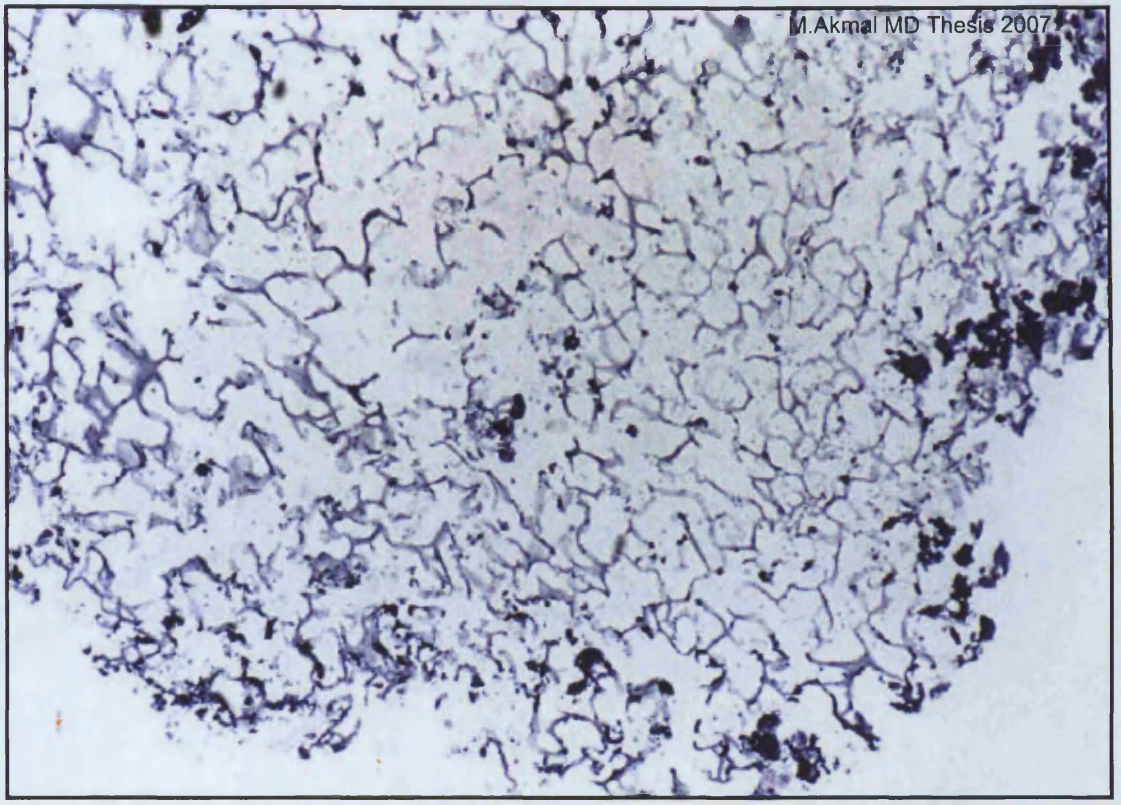


Figure 4.1.7 Magnification x4. H&E Stain.
Collagen/Alginate disc construct taken at Day 10. STATIC

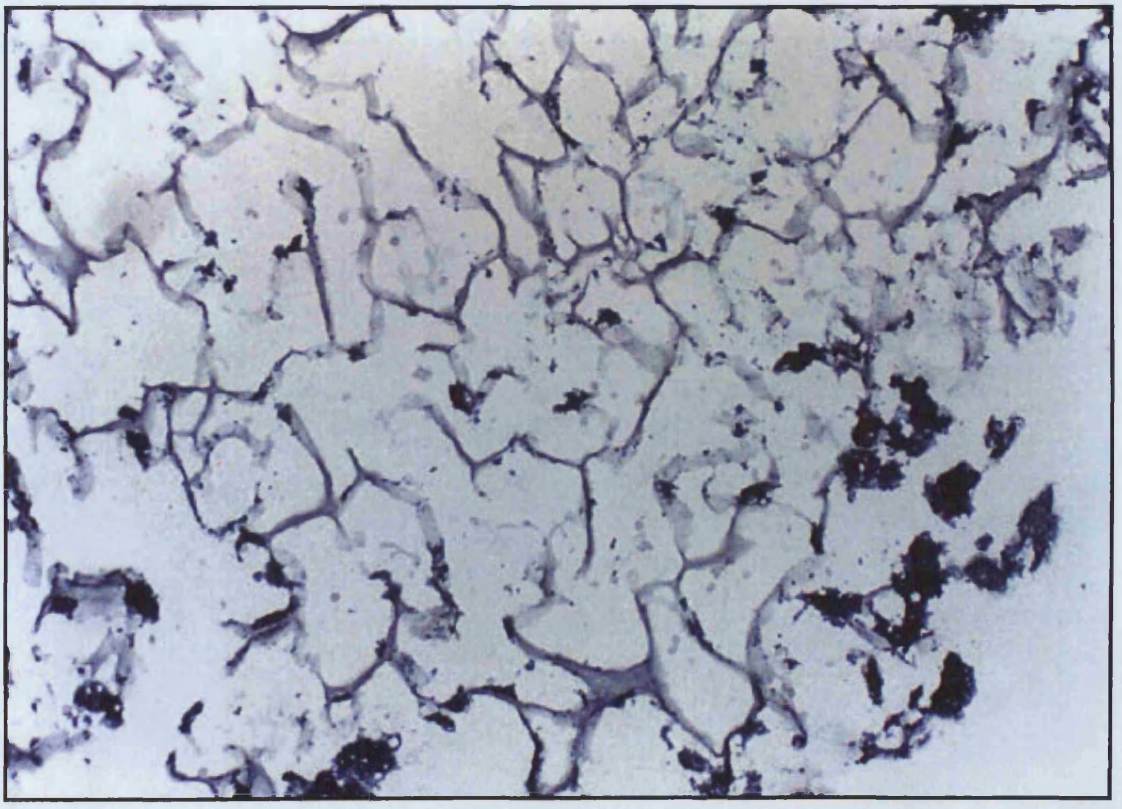


Figure 4.1.8 Magnification x10. H&E Stain.
Collagen/Alginate disc construct taken at Day 10. STATIC

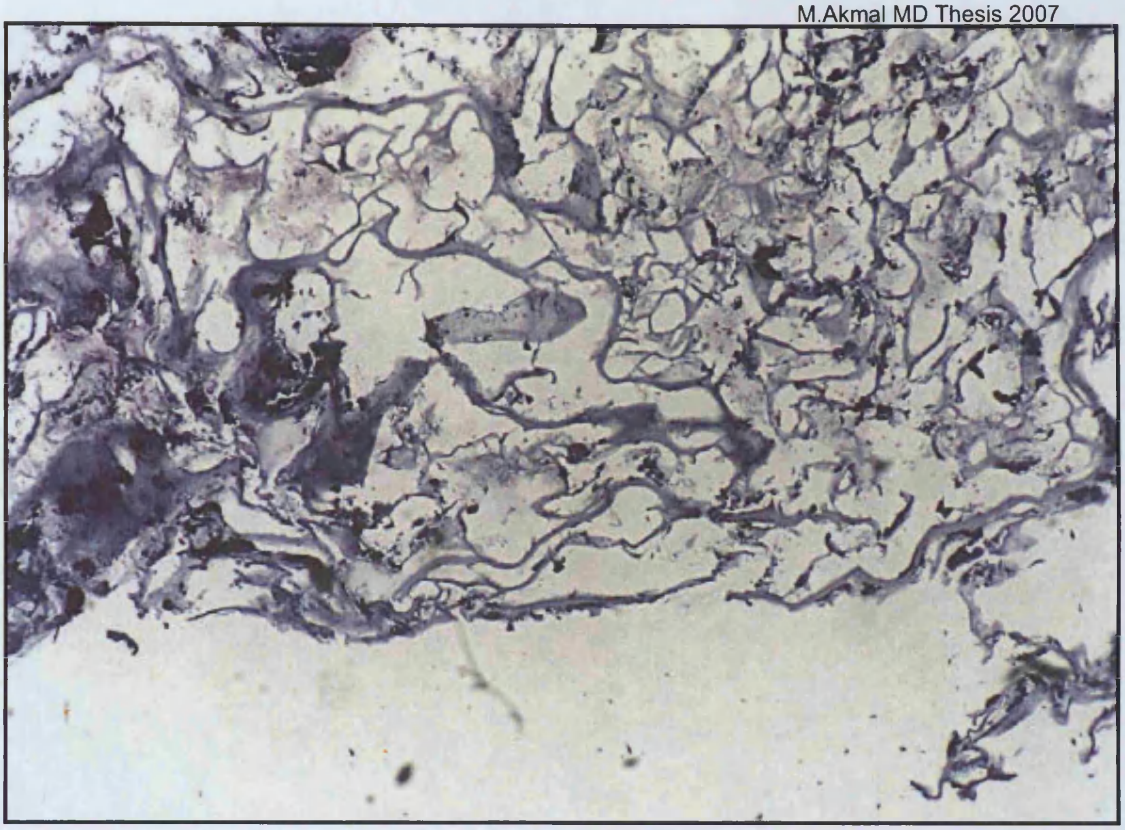


Figure 4.1.9 Magnification x4. H&E Stain.
Collagen/Alginate disc construct at day 10. ROLAMIXER

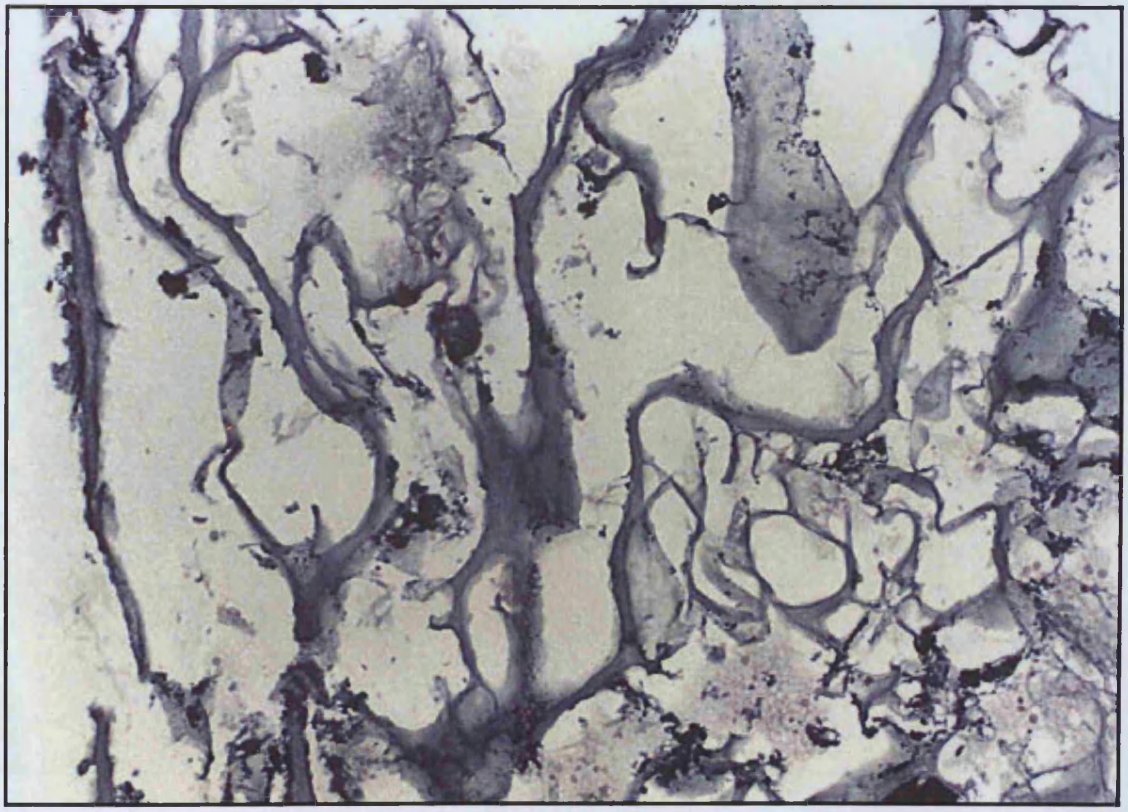


Figure 4.1.10 Magnification x10. H&E Stain.
Collagen/Alginate disc construct at day 10. ROLAMIXER

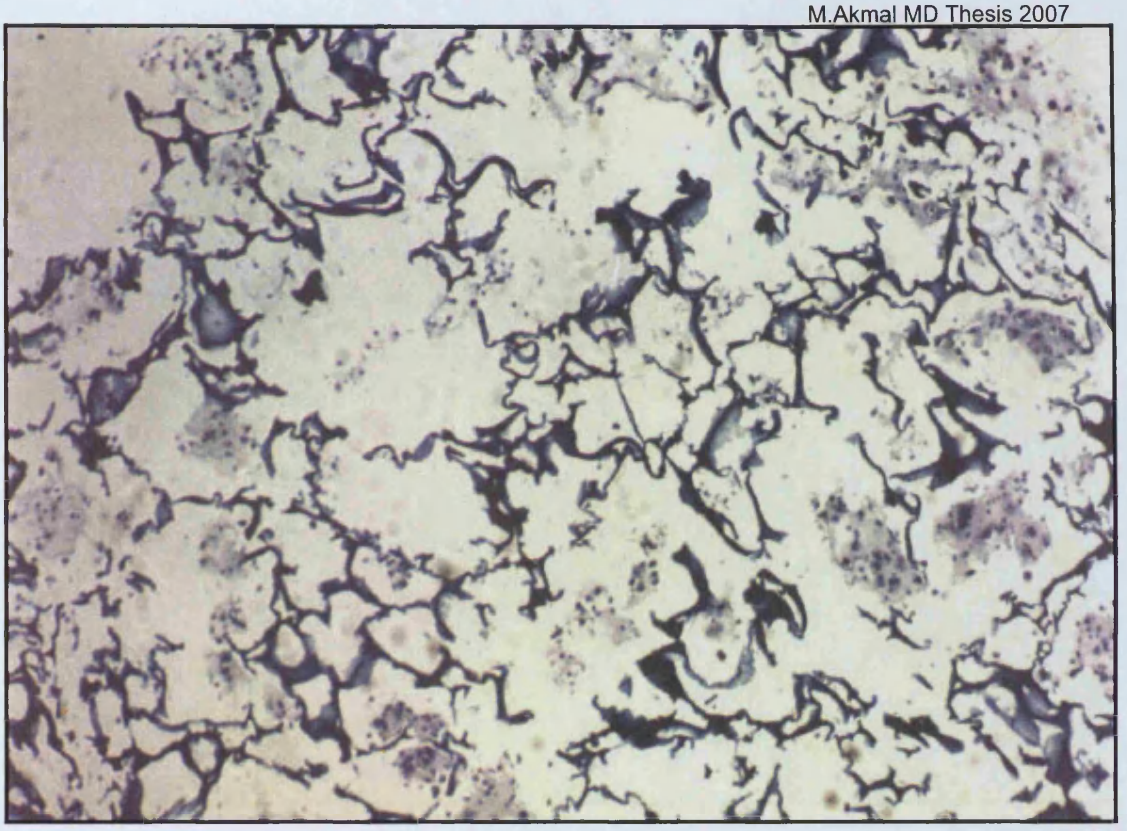


Figure 4.1.11 Magnification x4. H&E Stain.
Collagen/Alginate disc construct taken at Day 14. STATIC

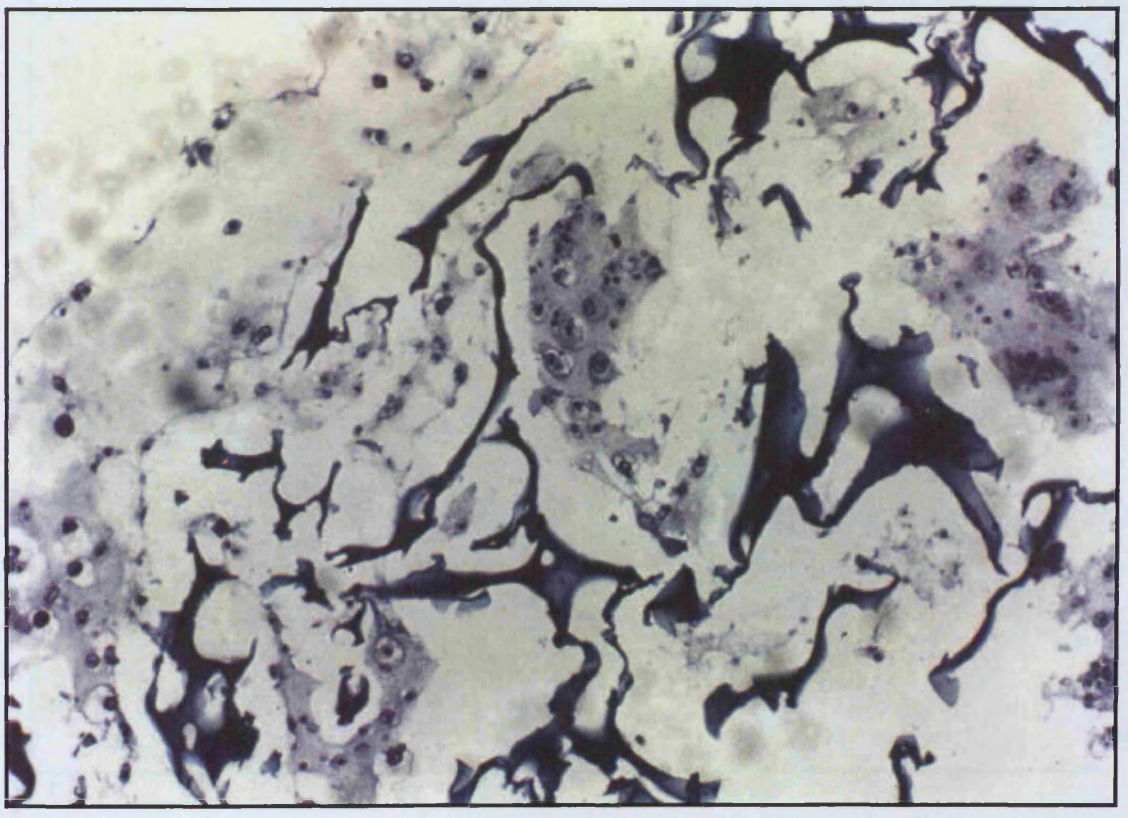


Figure 4.1.12 Magnification x4. H&E Stain.
Collagen/Alginate disc construct taken at Day 14. STATIC

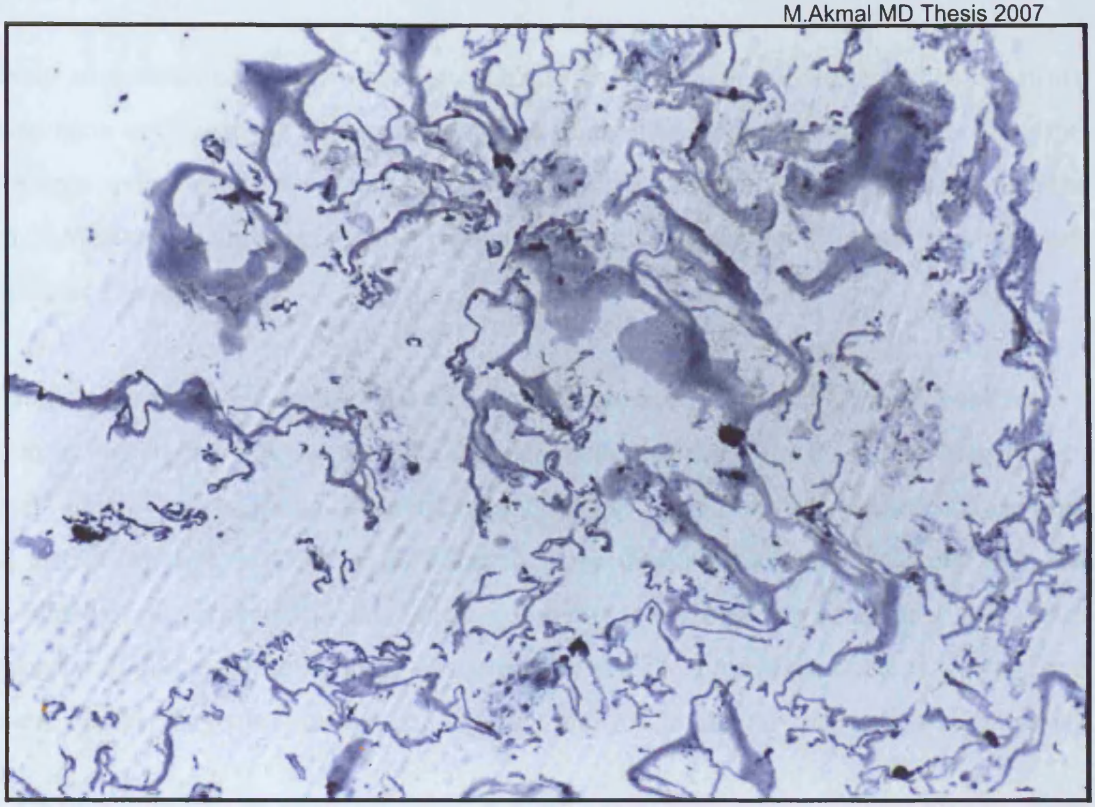


Figure 4.1.13 Magnification x4. H&E Stain.
Collagen/Alginate disc construct at day 14. ROLAMIXER

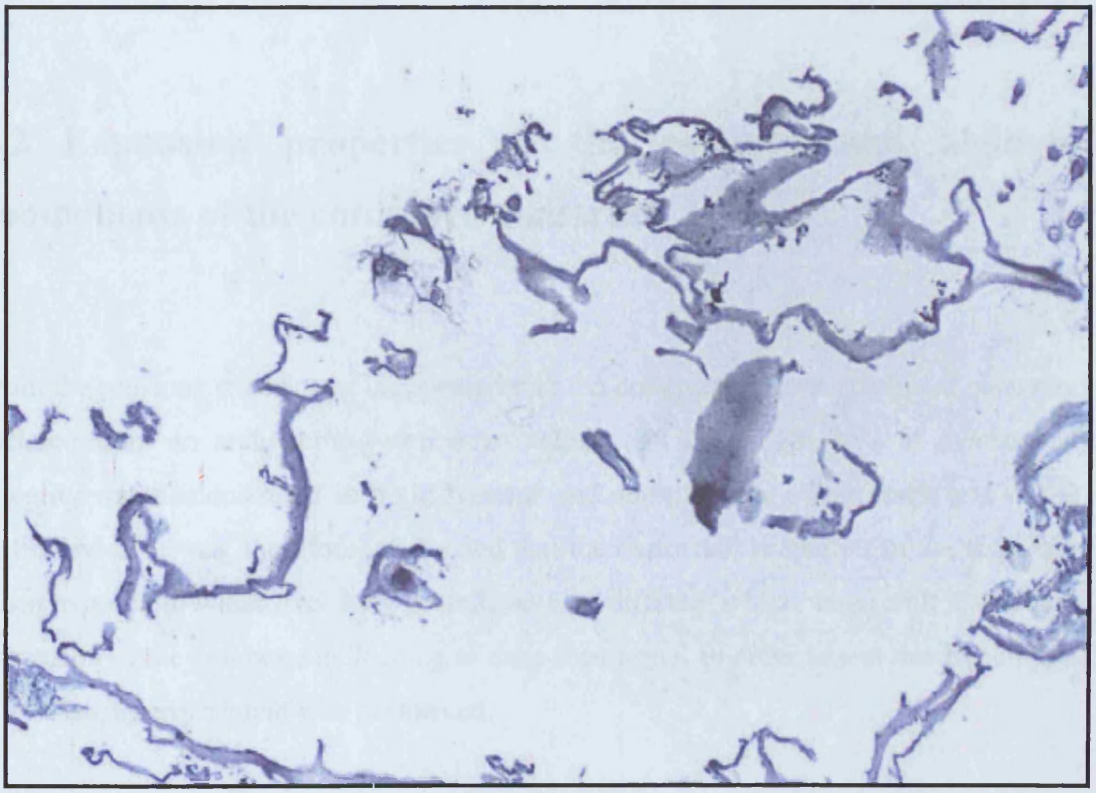


Figure 4.1.14 Magnification x10. H&E Stain.
Collagen/Alginate disc construct at day 14. ROLAMIXER

likely as a result of fluid absorption from the culture medium, which led to construct expansion and material fragmentation. The fragmented alginate was able to leave the collagen sponge and settle at the bottom of the culture bottles. As the chondrocytes were embedded within alginate, the loss of this material led to an overall loss of chondrocytes from the construct.

The findings were much more marked in the rolamixer cultures. The additional feature seen in this device was the considerable destruction of the collagen sponge architecture itself which was becoming apparent as early as day five of culture. Factors contributing to this destructive process in addition to those discussed above may have been the presence of numerous collisions between constructs and between constructs and culture vessel wall due to the rotation of the culture bottle. The high shear stresses resulted in a much quicker degeneration of the construct and led to almost complete destruction by day 14.

The presence of chondrocytes in static cultures at day 14 together with the presence of divided cells and matrix suggests that the culture conditions were suitable for cell replication.

4.2 Expansion properties of the collagen and alginate components of the combined construct

From the previous experiment it appeared that the collagen/alginate combined construct suffered from an architectural deficiency whilst in culture. The loss of mechanical integrity was demonstrated in both dynamic and static culture albeit much less in the static device. It was, therefore, postulated that the expansion properties of the materials when exposed to water over long periods may be different which may result in unequal expansion of the components leading to their separation. In order to test this hypothesis the following experiment was performed.

4.2.1 Protocol

Alginate and collagen were weighed at different stages of the construct forming and culture stages. The construct as a whole, and the individual components separately, were weighed at different time points during both formation and during culture. Graphs were plotted to show the changes in weight throughout a culture period. The changes in weight were caused by absorption of fluid from the culture medium and therefore a change in weight was indicative of a change in volume. It was assumed that there was a direct relationship between weight and volume and as we were comparing the materials with each other, the actual values were not so important as the relative values. Due to the considerably small size of the constructs and the variations in their shape when being weighed it was not possible to calculate the volume directly for each component.

Thirty five collagen sponge constructs were prepared by coring out discs from flat sheets of collagen sponge. The sponges were 6 mm diameter and 4 mm thick. The approximate volume for each dry sponge was therefore 113 mm^3.

Twelve collagen sponges were incubated in culture medium for one hour and then individually weighed wet in order to establish the mean initial wet weight of the collagen sponges. The sponges were then taken through the same procedure as collagen/alginate constructs during formation and weighing was done at time points of 20mins (ie immediately after the gelation stage with $CaCl_2$) 1hr, 12 hrs, 18hrs, 24hrs, 36hrs and 72hrs.

Five collagen sponges were weighed and incubated in 5 mls of 2% solution of low viscosity alginate. The sponges were weighed at the same time points as above to establish the change in weight of collagen sponge as it absorbed alginate.

Twelve collagen sponges were used to form collagen/alginate constructs. Following the one hour incubation period of the collagen/alginate combined construct they were individually weighed. The constructs were then gelled using $CaCl_2$ solution, washed in medium and then re-weighed at the 20 minute time point. The constructs were then cultured in a static culture dish and weighed at time points as above.

Ten, 100 µl alginate/chondrocyte solution samples were weighed to represent an initial time zero weight for the alginate component alone. The alginate/chondrocyte samples were then gelled using the same procedure as above and were again weighed at the same time points as above. 100 µl (ie 100mm^3) of the solution was chosen as this quantity represented the same quantity used in the formation of the collagen/alginate constructs. Therefore, we could make a direct comparison between alginate alone and the combined construct.

Three Collagen/Alginate constructs were collected at time point 36 hrs. The samples were fixed in paraformaldehyde and analysed histologically.

4.2.2 Results

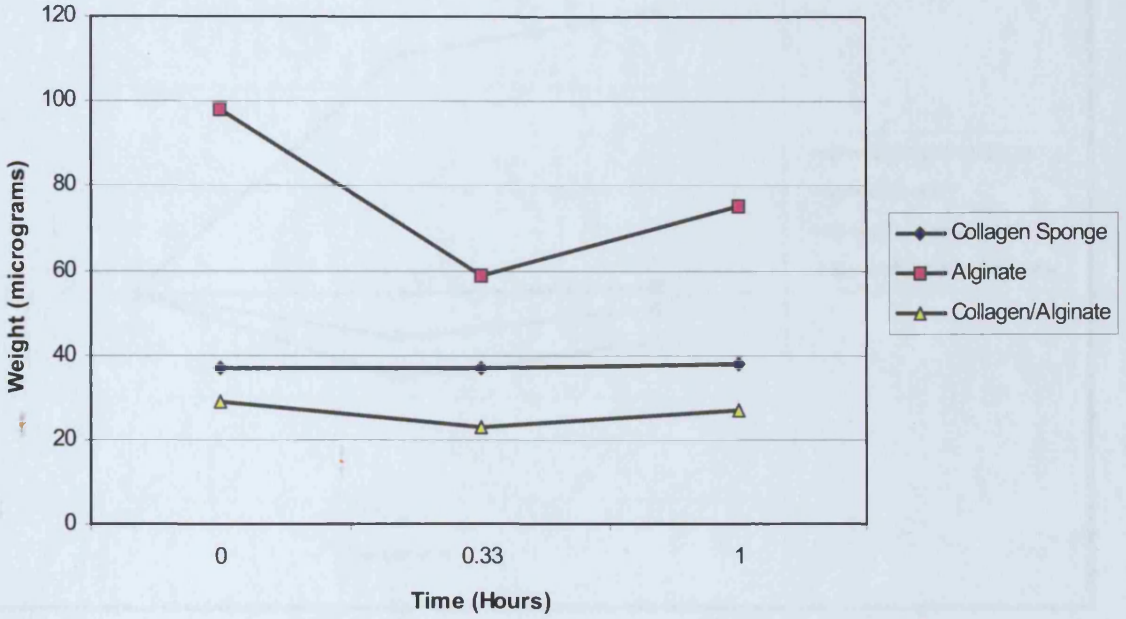
Table 1. All values are mean weights

Change in actual weight and % weight of constructs incubated under the same conditions in a static culture disc for 72 hours in medium

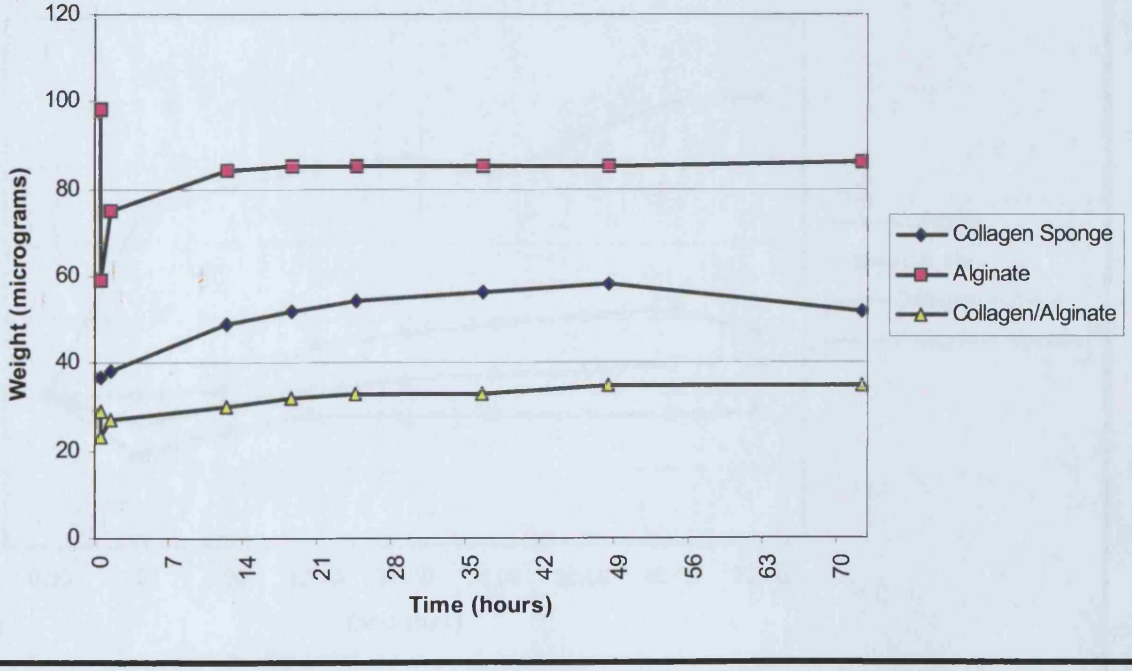
	Collagen Sponge		Alginate		Collagen/Alginate		Collagen sponge In alginate solution	
Time (hours)	Actual weight (micrograms)	% weight	Actual weight (micrograms)	% weight	Actual weight (micrograms)	% weight	Actual weight (micrograms)	% weight
0	37	100	98	100	29	100	36	100
0.33	37	100	59	60	23	79	78	216
1	38	103	75	77	27	93	86	238
12	49	132	84	86	30	103	90	250
18	52	141	85	87	32	110	93	258
24	54	146	85	87	33	114	88	244
36	56	151	85	87	33	114	98	272
48	58	157	85	87	35	121	106	294
72	52	141	86	88	35	121	107	297

The data from table 1 has been plotted out in graphical format

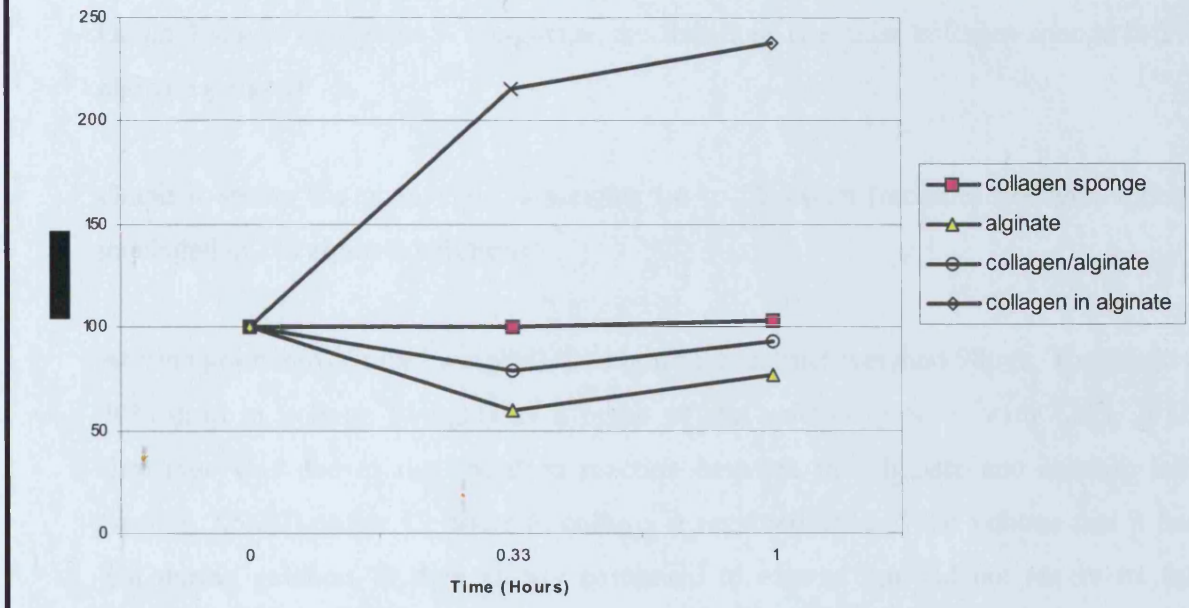
Graph 1.
Mean weights of 12 constructs composed of collagen only, collagen/alginate and alginate only incubated in culture medium for upto 72 hours (first hour shown)



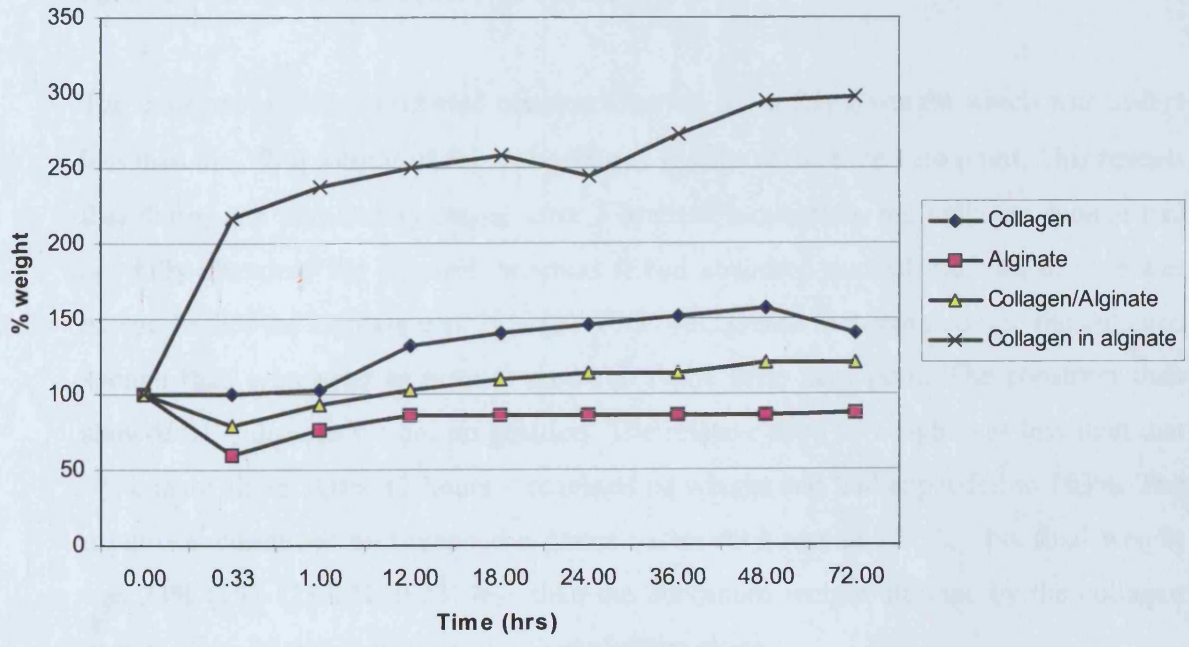
Graph 2.
Mean weights of 12 constructs composed of collagen only, collagen/alginate and alginate only, incubated in culture medium for upto 72 hours



Graph 3.
Percentage of original weight of 12 constructs composed of collagen only, collagen/alginate and alginate only, incubated in culture medium and a collagen sponge incubated in alginate solution for upto 72 hours (first hour shown)



Graph 4.
Percentage of original weight of 12 constructs composed of collagen only, collagen/alginate and alginate only, incubated in culture medium and a collagen sponge incubated in alginate soultion for upto 72 hours



Graph 1 shows the change in actual weights in the first hour

Graph 2 shows the change in actual weights up to 72 hours

Graph 3 shows change in % weights in the first hour (Includes collagen sponge in 2% alginate soution)

Graph 4 shows the change in % weights up to 72 hours (includes collagen sponge incubated in 2% alginate solution)

At time point zero (initial weights) the alginate construct weighed 98µgs. There was a 40% drop in volume (weight) as a result of the gelation process with $CaCl_2$. This shrinkage was due to the chelation reaction between the alginate and calcium ions (section 2.5.2.3). After 12 hours in culture, it regained 25% of the volume that it had lost during gelation. It then slowly continued to expand but did not regain its full volume and even after 72 hours in culture it reached a plateau of only 88% of its original weight.

The collagen sponge, as expected, on its own showed no change in volume during the gelation process because $CaCl_2$ has no effect on collagen. It gradually expanded by 32 % of its original weight by 12 hours. In 36 hours it reached a maximum weight of 157% but then lost weight to become 141% at 72 hours.

The collagen/alginate combined construct started off at 29µg weight which was in fact less than the 37µg weight of the wet collagen sponge at the time zero point. This reveals that during the preparatory stages after 1 hour of incubation, the collagen sponge had not fully absorbed the alginate, whereas it had absorbed normal medium to a greater extent. In fact the alginate was 22% (37-29/37=0.22) less incorporated into the collagen sponge than compared to normal medium at the time zero point The construct then showed 21% drop in weight on gelation. The relative drop in weight was less than that of alginate alone. After 12 hours it regained its weight and had expanded to 103%. The expansion continued and reached a plateau after 48 hours at 121%. This final weight was 23% (157-121/121=0.23) less than the maximum weight attained by the collagen sponge alone but was much greater than alginate alone.

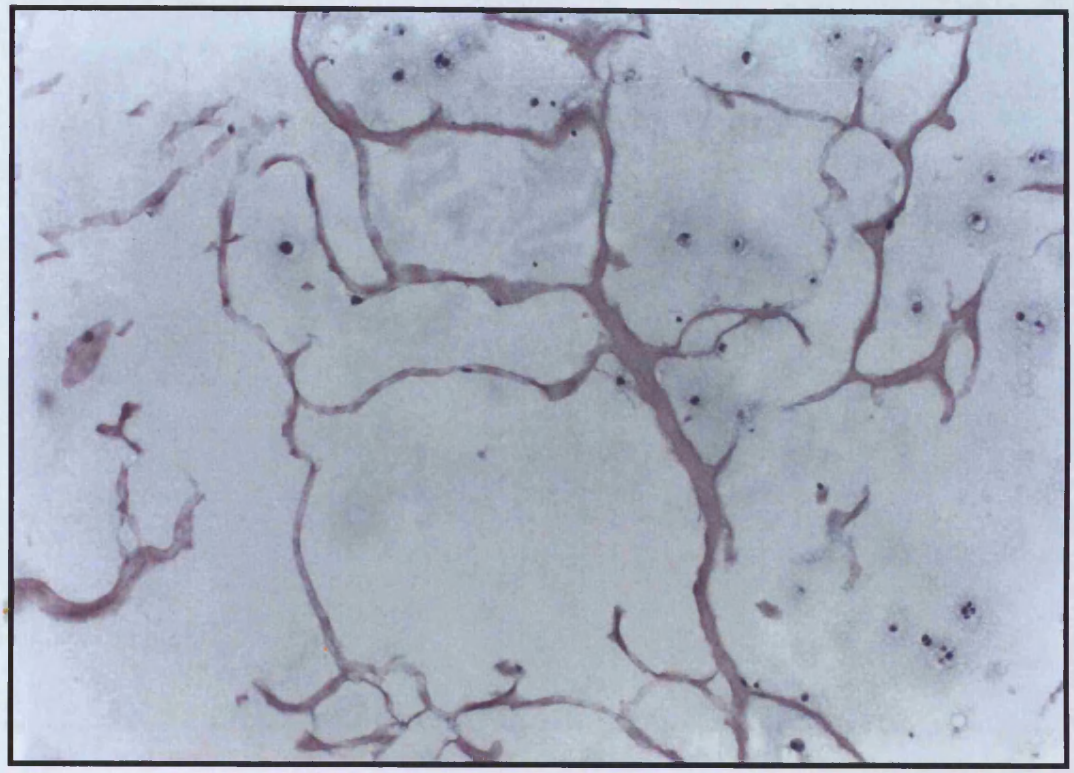


Figure 4.2.1 Magnification x10. Ehrlichs H&E stain collagen/alginate disc construct taken after 36 hrs incubation

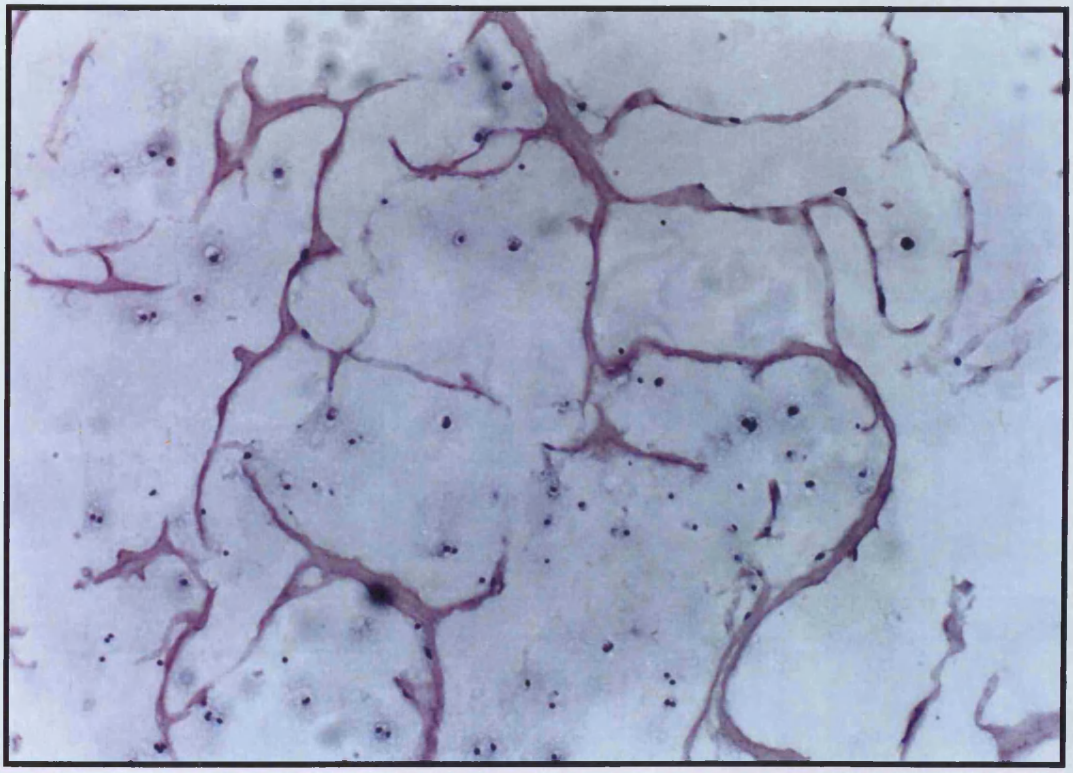


Figure 4.2.2 Magnification x10. Ehrlichs H&E stain collagen/alginate disc construct taken after 36 hrs incubation

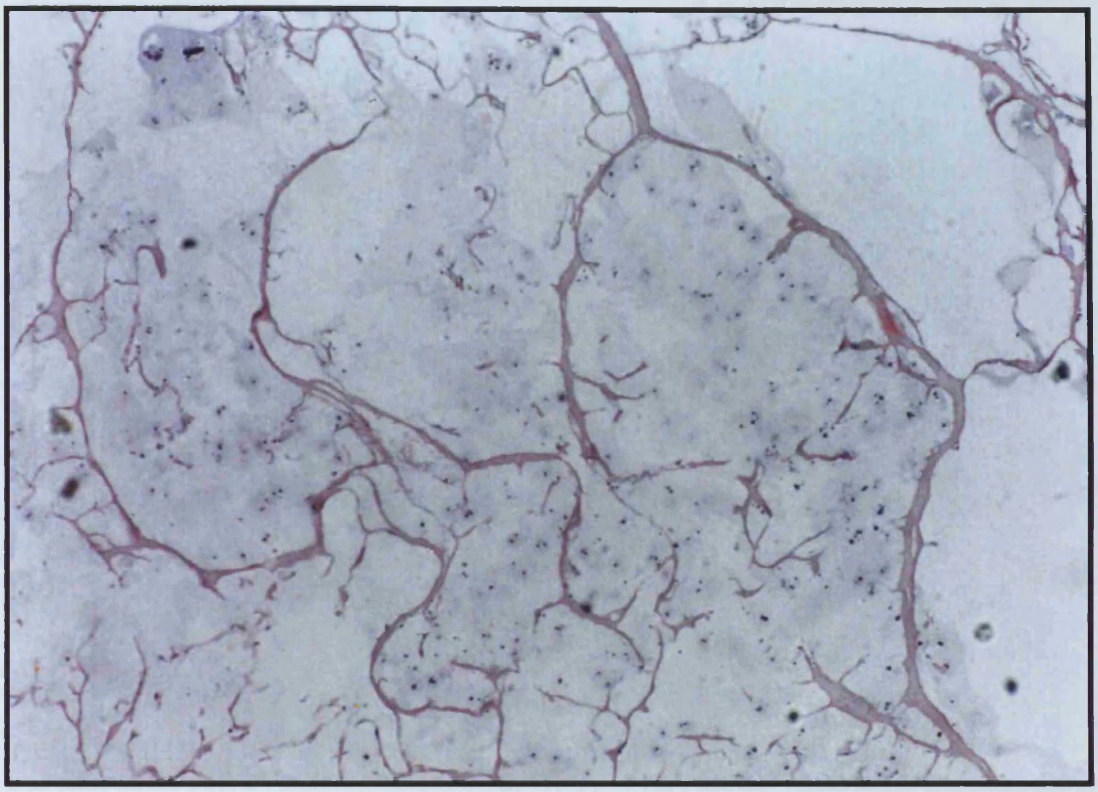


Figure 4.2.3 Magnification x4. Ehrlichs H&E stain collagen/alginate disc construct taken after 36 hrs incubation . Alginate fragments within collagen sponge meshwork

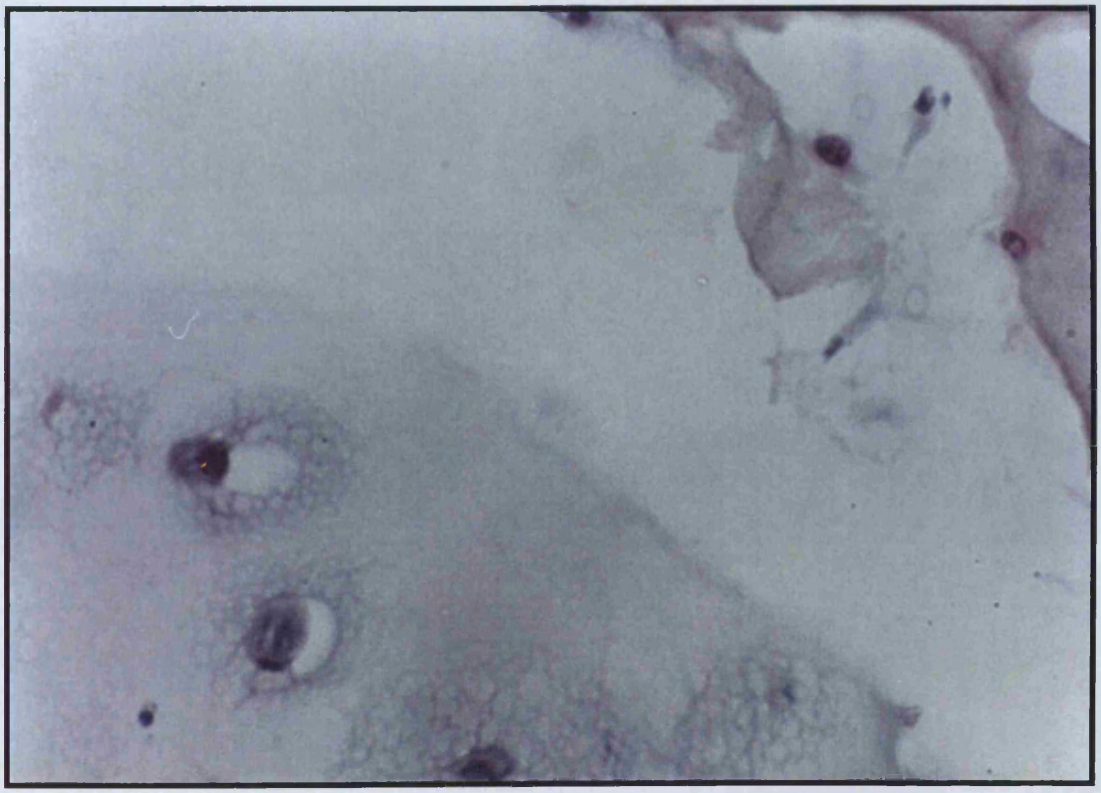


Figure 4.2.4 Magnification x40. Ehrlichs H&E stain collagen/alginate disc construct taken after 36 hrs incubation. Alginate peeling away from collagen fibres

The collagen sponge incubated in alginate solution had a weight of 36μg at the 1 hour time point. However, if the incubation of collagen in the alginate solution continues then the collagen sponge continues to absorb alginate. After only a further 20 minutes it doubles its weight to 216% which continues to increase to a plateau of 108 μg which is 297% of the original weight. In fact the final weight after 72 hours is most likely due completely to the alginate solution as the collagen sponge does not itself weigh much. It was notable that the collagen sponge incubated in alginate reaches a much higher weight than if incubated in medium.

Figure 4.2.1 shows a collagen/alginate disc construct at 36 hours culture time point. There was a poor penetration of alginate and chondrocytes into the central regions of the construct (bottom left). In the peripheral areas (top right) there was good penetration of both alginate and chondrocytes.

Figure 4.2.2 shows a peripheral region of a collagen/alginate disc construct at 36 hours which had incorporated the alginate well. There was good alginate and chondrocyte distribution. Some chondrocytes even had small zones of matrix developing around them. This was suggestive of suitable conditions for chondrocyte culture. However, these areas were very limited.

Figure 4.2.3 shows the typical appearance of fragmentation of regions of alginate occurring. The alginate was becoming separated from the collagen fibres.

Figure 4.2.4 shows the effect of alginate detaching from the collagen fibres at higher magnification. The space between alginate and collagen fibres was occupied by culture medium.

4.2.3 Conclusion

The collagen/alginate construct that was under investigation in these experiments has a number of potential fundamental problems and its use in cartilage tissue engineering is seriously limited. The 1 hour period of incubation of the collagen with alginate solution is not sufficient to allow a full incorporation of alginate into the collagen sponge. This is reflected in the results from the collagen sponge incubation in alginate solution. It takes up to 12 hours for the collagen sponge to absorb 80% of its maximum capacity of

alginate at 2% concentration. This may be due to the higher viscosity of alginate as compared to medium. These results also explain the histological findings of alginate being concentrated around the periphery and less alginate penetrating deep within the construct. Attempts to use suction methods to incorporate the alginate into the collagen sponge have been tried by my predecessor but were met with failure as the collagen sponge architecture was destroyed.

The volume expansion characteristics of the collagen sponge and the alginate gel are completely different during gelation and also during incubation. The collagen sponge expands gradually from the time of wetting whereas the alginate shrinks on gelation and never quite reaches its normal volume again. The collagen sponge and alginate are constantly opposing each other.

The histological analysis supports the above findings, showing an incompatibility between the two components in that they oppose each other in expansion during gelation and when incubated in culture medium. This obviously has an effect of mechanically weakening the construct as a whole and it leads to a gradual destruction and loss of chondrocytes and alginate from the system. The collection of white clumps of alginate at the bottom of the culture bottles was a direct result of these detrimental processes within the construct.

Chapter V

Agarose Constructs in a Bioreactor

Experiments using the Rotating Wall vessel to establish whether dynamic culture systems may improve cell culture

The experiments so far were conducted in order to further characterise the collagen/alginate construct and assess whether it would be suitable for use as a scaffold for chondrocyte transfer in the aim of repairing articular cartilage defects. The construct was also assessed for its potential to be used in investigation of dynamic culture systems. The collagen/alginate construct although offering a suitable environment for chondrocyte culture, suffers considerably from its mechanical weakness when subjected to culture medium and its gradual physical destruction makes biochemical quantification of matrix and cell components invalid. More importantly in relation to dynamic culture systems, the direct comparison with other static systems is not possible. In order to interpret DNA and GAG changes, it is important to have a construct which does not deteriorate and lose large amounts of material while in culture. As the second part of this research project was to investigate methods of improving mass transfer of nutrients to cells in the centre of constructs it was not satisfactory to use the collagen/alginate construct for these experiments.

Agarose as discussed earlier in section (introductory chapter on agarose) is a well characterised construct material which has mechanical properties which would be suitable for dynamic culture systems. It supports the culture of chondrocytes well and provides a good three dimensional architecture.

The following experiments were therefore conducted using agarose.

5.1 Use of agarose disc scaffolds in the Rotating Wall vessel.

In this experiment agarose constructs containing chondrocytes cultured under either static conditions in a culture dish or dynamic conditions in a rotating wall vessel for twelve days were compared. Biochemical analysis for DNA and GAG synthesis and histological analysis was performed at time points of zero, two days, seven days and twelve days. The static system was compared to the dynamic system in relation to cell proliferation and matrix production.

5.1.1 Protocol

Fourty five agarose disc constructs containing a chondrocyte concentration of 10 million cells per millilitre of agarose were formed using techniques described in the methods section. All procedures were performed under strict aseptic conditions and all materials used were freshly prepared. The chondrocytes were harvested from bovine metacarpo-phalangeal joints of no longer than 6 hours of slaughter

Twenty discs were carefully added into the culture chamber of the rotating wall vessel. The culture chamber was filled with 50mls of freshly prepared culture medium. The chamber was assembled on to the base unit and the whole instrument placed in a 37°C, 5%CO_2 incubator. The speed of rotation was adjusted until all agarose disc constructs were suspended in the medium and were in a state of free fall.

Twenty discs were placed in a sterile culture dish with 50 mls of fresh culture medium. The disc constructs settled at the bottom of the flat culture dish and were left undisturbed. The culture dish was then also placed in the incubator.
Three disc constructs were collected separately into 2ml sterile biochemical analysis tubes for biochemical analysis. Two constructs were collected in 20mls paraformaldehyde in sterile containers and kept in a 4°C fridge for histological analysis. These specimens represented day zero samples.

Medium was changed and replaced with fresh culture medium at 48 hour intervals in both the static and dynamic culture systems. Samples were collected at time points of 2 days, 7 days and 12 days. An additional medium change was performed on these days

when specimens were collected. Three samples were collected at each time point from each system for biochemical analysis and two samples were collected at each time point for histological analysis.

An additional two discs at the twelve day time point were collected separately in 2mls of fresh medium. One disc was labelled with ethidium homodimer and Calcein am and examined under the fluorescent microscope to assess the distribution of live cells and dead cells. Photographs were taken of representative regions. The other disc was examined directly using an Olympus microscope. Photographs using the contrast filter setting were taken of representative areas of the construct. Spare disc constructs were present in each system to replace any constructs that were damaged during the handling procedures.

DNA and GAG analysis was performed as a batch on all samples and results were tabulated and plotted using a Microsoft excel spreadsheet. Students T-Test was used to calculate the statistical significance of any differences. After processing and sectioning of all samples collected for histology, H&E and Safranin-O staining was performed. Representative sections were photographed using an Olympus microscope.

5.1.2 Results

5.1.2.1 Biochemistry

Table 2

Mean DNA concentration of agarose constructs cultured in a RWV and a Static culture dish for upto 12 days.

	RWV		PETRI	
Day	DNA Conc µg/ml	SEM	DNA Conc µg/ml	SEM
0	40	2	40	2
2	42	1	45	2
7	58	3	54	1
12	120	4	86	2

SEM = Standard Error of the Mean

Graph 5 and Table 2 show the mean DNA concentration per millilitre of agarose construct at the different time points. The initial concentration of DNA per construct was 40.4 $\mu g.ml^{-1}$ which corresponds to a cell number of 6.7 million ($40.4 \times 10^{-3} / 6 \times 10^{-9}$) cells per ml of construct as each chondrocyte contains approximately 6.7×10^{-9} $\mu g.ml^{-1}$. Although an attempt was made to make disc constructs contain 10 million cells per ml, the manual cell count method used in the cell isolation stages was not as accurate as the biochemical method and therefore errors were inevitable. The lower cell numbers however did not effect the results as any changes in DNA concentration were compared to the initial starting concentration .

Although there was no significant increase in the DNA concentration in both systems at day 2, by day seven, there was a significant increase to a value of 58 $\mu g.ml^{-1}$ in the RWV and 54 $\mu g.ml^{-1}$ in the static device. This represents a 43% increase in the RWV and a 32% increase in the static system. The difference between the two culture systems was not significant ($p>0.05$)

At day 12, the DNA increases were significant. The constructs cultured in the RWV showed a DNA concentration of 120 $\mu g.ml^{-1}$ and the static system 86 $\mu g.ml^{-1}$ representing an increase in DNA concentration of 198% and 114% respectively. The difference between the two systems was also significant ($p<0.05$ Students T-Test) at day 12.

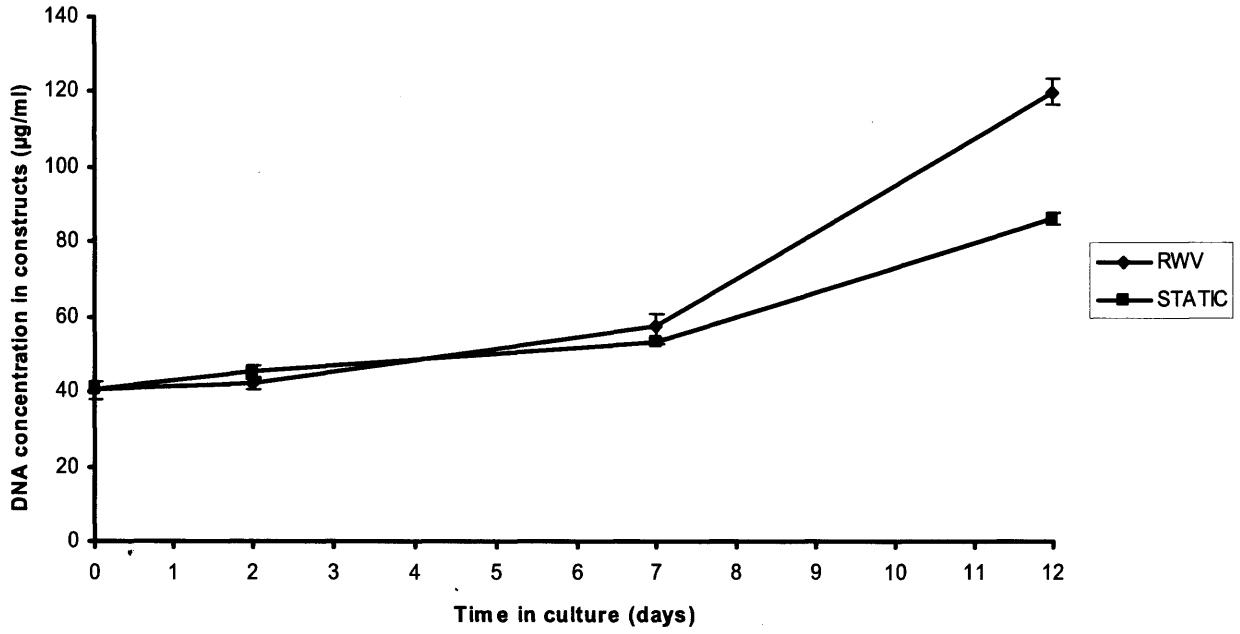
Table 3

Mean GAG concentration of Agarose constructs cultured in a RWV and a Static culture dish for up to 12 days.

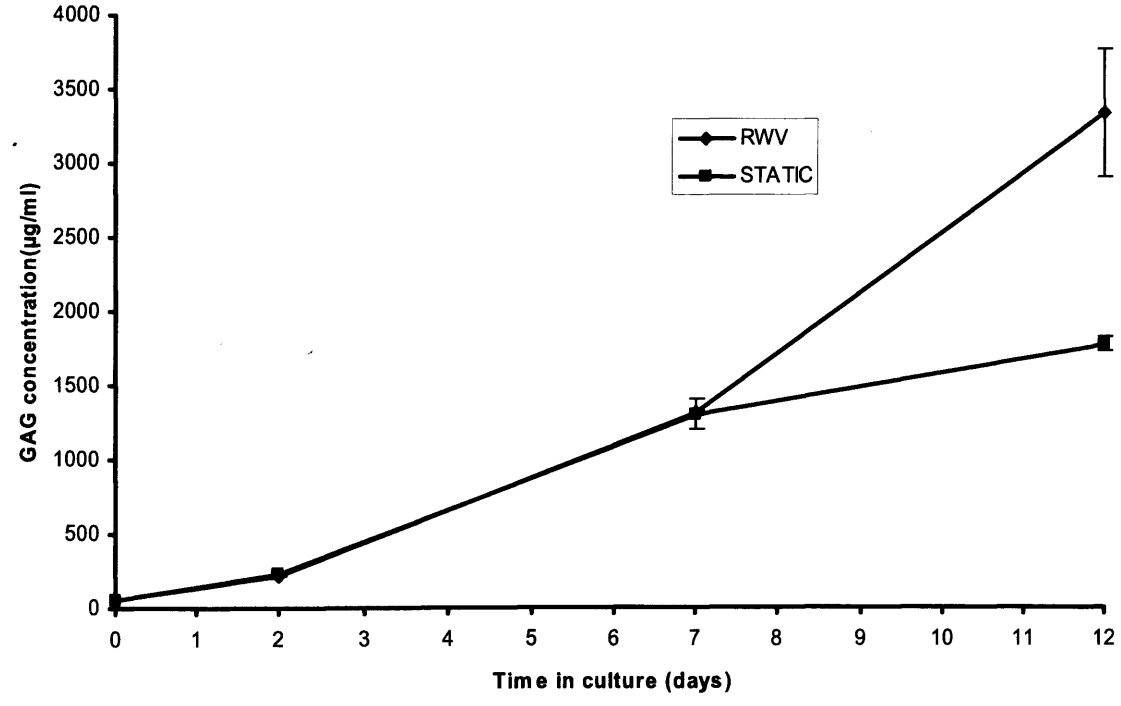
	RWV		PETRI	
Day	GAG Conc ($\mu g.ml^{-1}$)	SEM	GAG Conc ($\mu g.ml^{-1}$)	SEM
0	60	4	60	4
2	215	4	234	14
7	1313	13	1301	100
12	3338	431	1775	50

SEM = Standard Error of the Mean

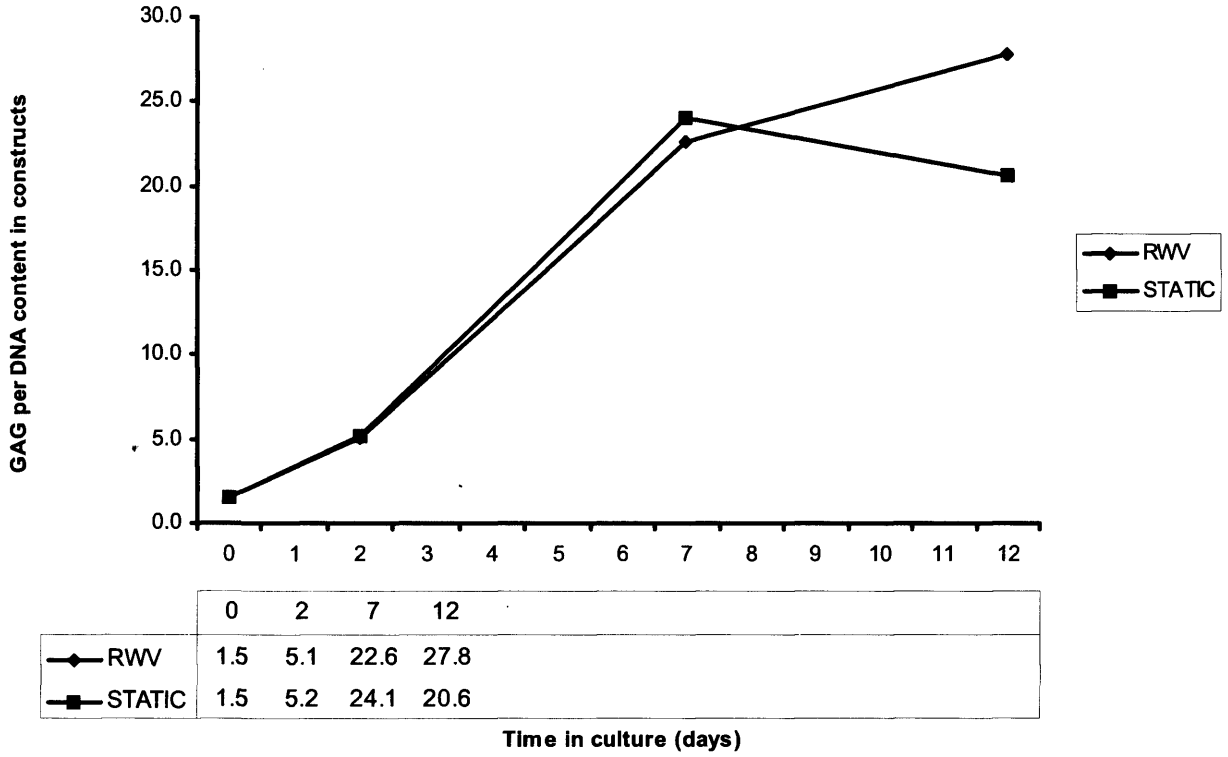
GRAPH 5. DNA content of cell seeded agarose disc constructs cultured for 12 days in a rotating wall vessel bioreactor and a static culture dish.



GRAPH 6. GAG content of cell seeded agarose disc constructs cultured for 12 days in a rotating wall vessel bioreactor and a static culture dish



GRAPH 7. GAG per DNA content of cell seeded agarose disc constructs cultured for 12 days in a rotating wall vessel bioreactor and a static culture dish.



	0	2	7	12
RWV	1.5	5.1	22.6	27.8
STATIC	1.5	5.2	24.1	20.6

Graph 6 and Table 3 show the mean GAG concentration per millilitre of agarose construct at the different time points of the experiment. The GAG concentration in the disc constructs at the start of the experiment was 60 $\mu g.ml^{-1}$. Although the cell isolation procedure removes most matrix components, it is likely that there still remains a small amount closely associated around the cell membrane of most cells. Even by day 2, there was a significant increase of GAG in both systems to a value of 234 $\mu g.ml^{-1}$ in the RWV and 215 $\mu g.ml^{-1}$ in the Static device. The GAG concentrations continue to increase and by day seven levels of 1.3 $mg.ml^{-1}$ were attained. However there was no significant difference between the two culture systems at this stage.

By day fourteen, a significant difference ($p<0.02$) between the two systems became apparent. The RWV cultured constructs contained a mean GAG concentration of 3.34 $mg.ml^{-1}$ whereas in the static device they had only attained 1.78 $mg.ml^{-1}$.

Graph 7 illustrates the GAG content per DNA during the culture period. Upto day 7, the ratio increases uniformly in both culture systems and implies a steady amount of GAG production by the cells. By day seven the ratio of GAG/DNA has increased to 23 in the RWV and 21 in the Static system. After day 7, cell proliferation rate increases significantly in the RWV but GAG rates do not increase correspondingly. Despite an increase in DNA and GAG, the overall ratio of GAG to DNA falls, implying cell proliferation rather than GAG production, during this phase of culture in the RWV.

5.1.2.2 Histology

Figures 5.1.1 and 5.1.2 show the peripheral margins of an agarose disc construct at day zero time point. The chondrocytes are well distributed within the agarose. There are no cell clusters and no evidence of extracellular matrix (ECM). *Figure 5.1.3* is a higher magnification showing individual chondrocytes composed of a nucleus and cytoplasm (this colours have an abnormal appearance due to an abnormal filter on the lens of microscope). The chondrocytes appear rounded and healthy.

Figures 5.1.4 and 5.1.5 show chondrocytes in agarose after 2 days in static culture. There is an area of ECM surrounding each chondrocyte. *Figure 5.1.4* shows

chondrocytes detached from the agarose construct. The cause of this is most likely a processing artefact. However, it may be possible that chondrocytes are breaking free from the agarose construct as their matrix expands beyond the edge of the construct. Once the matrix penetrates the outer margin of the construct, it may be causing cells to literally fall out into the surrounding medium.

Figures 5.1.6 and 5.1.7 show chondrocytes after 2 days in dynamic culture. The differences between the two systems are obvious, there is more matrix surrounding the cells than in the case of the static system. The matrix appears to have 2 zones, an inner less intensely staining zone which corresponds to the pericellular matrix and an outer more intensely staining zone corresponding to the territorial matrix. There is no evidence of cell division in either system at this stage.

Figures 5.1.8 and 5.1.9 show chondrocytes located around the edges of a construct cultured for 7 days in the static device. The majority of cells remain singular and appear to have more matrix around their margins than at day 2. There are the appearances of a third zone surrounding the inner two zones, this corresponds to the inter-territorial matrix. The clarity between the zones is much more obvious than at day 2. The other obvious feature is the presence of a monolayer of chondrocytes around the margins of the construct. The monolayer is approximately 1 cell layer thick and appears to coat the construct completely. *Figure 5.1.10* is a section from the centre of the disc construct and shows a smaller area of matrix around the cells and the chondrocytes appear small with dark nuclei. They do not appear as healthy as the more peripherally located cells.

Figures 5.1.11 and 5.1.12 show chondrocytes located around the edge of a construct cultured for 7 days in the RWV. The majority of cells have divided to form spindle shaped cell clumps. The matrix has organised around the cell clumps and they have formed a single unit. The lightly stained interterritorial matrix is also visible around the outer margins of the cell clumps. There is no appearance of a monolayer around the margins of the construct as in the static culture. *Figure 5.1.13* is a section from the centre of the disc construct and shows cells with a smaller region of surrounding matrix, however there is more matrix than the equivalently located cells in static culture. The centrally located cells also appear more healthy and less pyknotic than in the static culture.

Figures 5.1.14, 5.1.15 and 5.1.16 show the margins of a construct cultured in the static culture dish at day 12 at different levels of magnification. The monolayer of cells has become multilayered and some of the cells within the agarose construct have now divided to form cell clumps. There is matrix associated with these cell clumps. However there are still singular cells present around the outer edges of the construct and some of these cells appear pyknotic and do not have any associated matrix. On a higher magnification it appears that the multilayer of cells have no associated staining matrix.

Figures 5.1.17, 5.1.18 and 5.1.19 show the margins of a construct cultured in the RWV at day 12 at different levels of magnification. The situation is significantly different from the statically cultured constructs. There are large spindle shaped cell clumps which often consist of 10 or more cells. The three layers of matrix are identifiable and in some places the inter-territorial matrix of neighbouring clumps has combined. There is no surface layer of multiple cells as in the case of the static system. There does not appear to be any particular direction in which the clumps form.

Figures 5.1.20 and 5.1.21 are sections taken from the centre of constructs cultured in a static device and a RWV respectively. In the static device centrally located cells appear very pyknotic with very little associated matrix. There is also very little evidence of cell division. However in the RWV cultured constructs, there are cells which are dividing and in some areas there a clumps of 3 and 4 cells. The matrix around the cells is more pronounced than in the static system. The underlying differences however between the peripherally located cells and the more centrally located cells are significant in both systems. The peripherally located cells appear more healthy showing cell division and greater matrix production than the centrally located cells.

Figure 5.1.22 is a phenomenan noticed around some areas of the RWV cultured constructs. It shows a clump of cells attached to the margins of the agarose, these cell clumps appear frequently around the margins and are only found in the RWV samples. *Figure 5.1.23* is a high magnification image of a centrally located chondrocyte in the RWV culture system. There is intense staining of matrix around the cell with the cell itself being located in the middle of the matrix and and having a relatively healthy appearence.

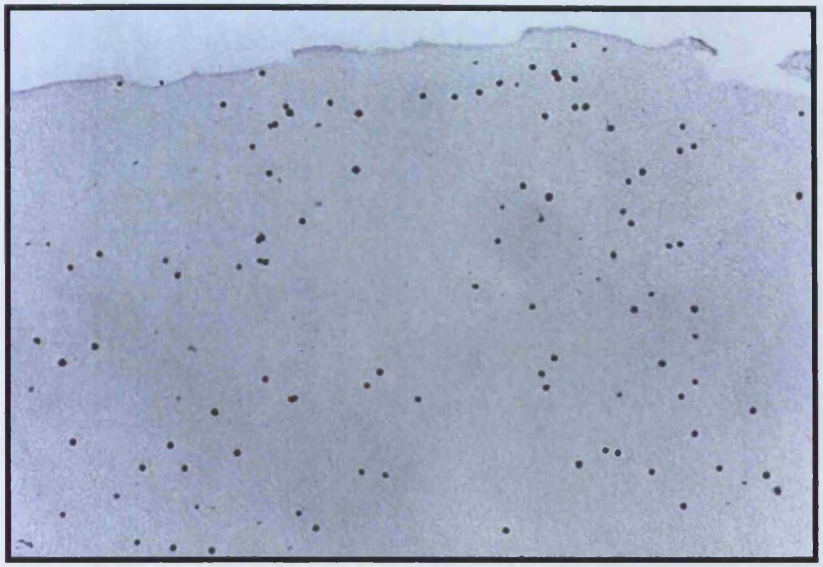


Figure 5.1.1
H&E Stain Magn X10

Day zero edge of agarose disc construct containing chondrocytes

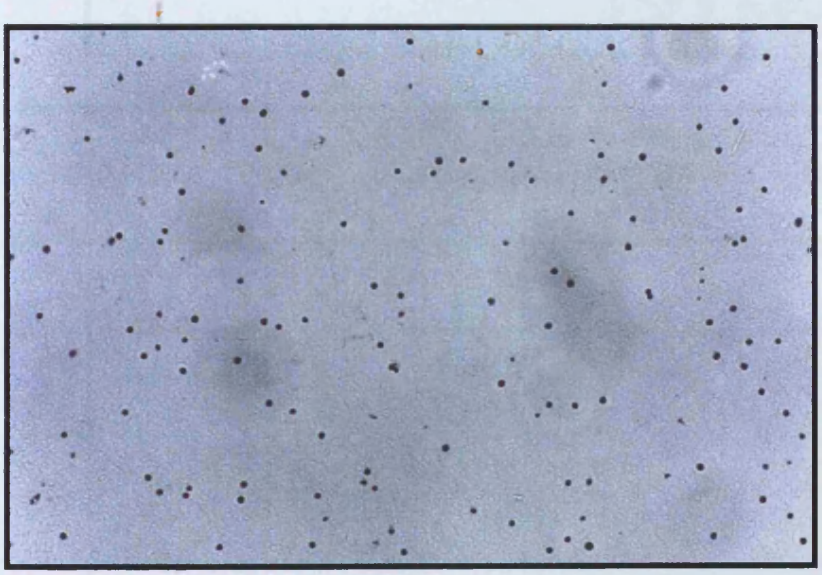


Figure 5.1.2
H&E Stain Magn X10

Day zero centre of agarose disc construct containing chondrocytes

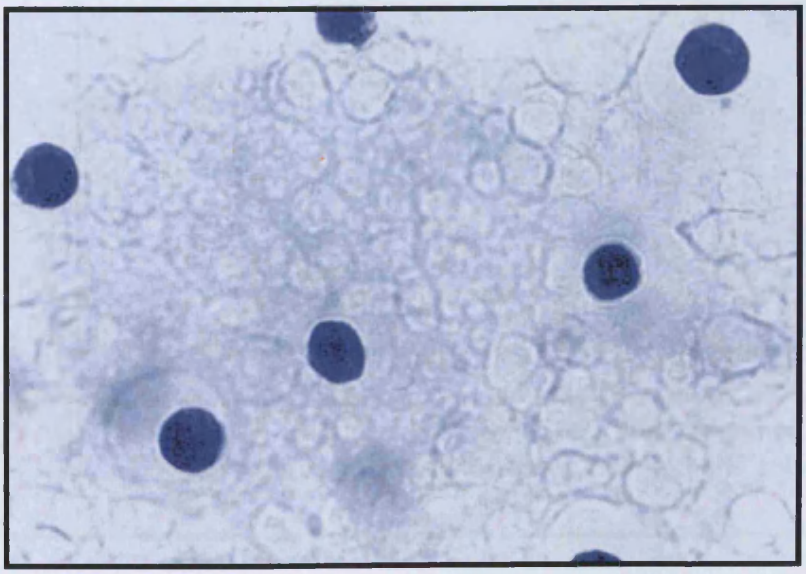


Figure 5.1.3
H&E Stain Magn X100

Day zero centre of agarose disc construct containing chondrocytes

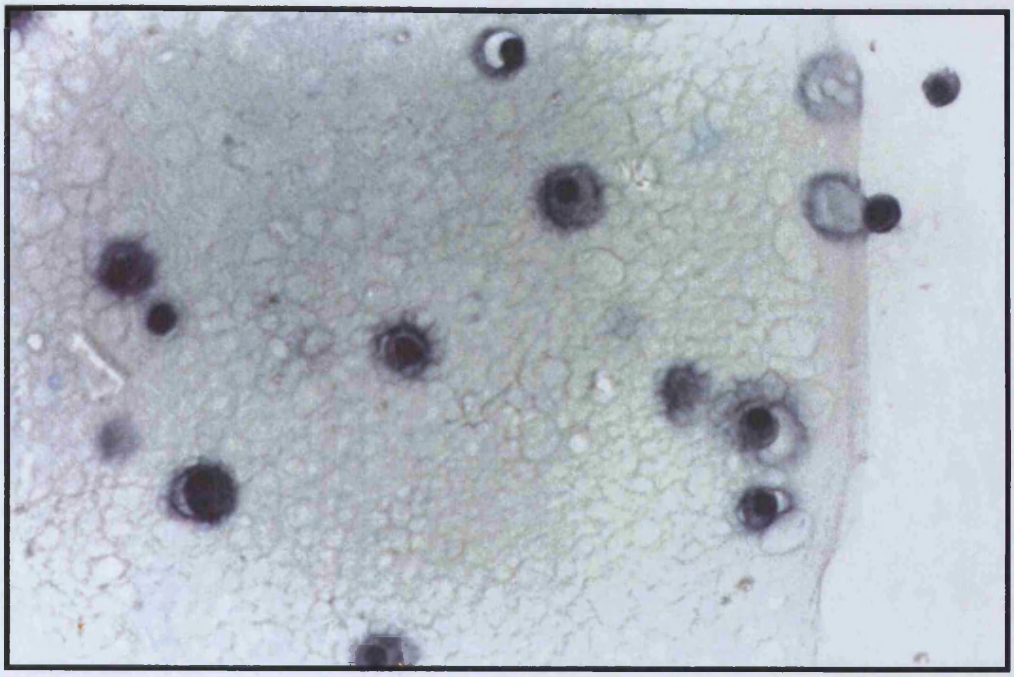


Figure 5.1.4 H& E Stain. Magnification X40.
Edge of Agarose disc construct cultured in Static culture dish for 2 days.

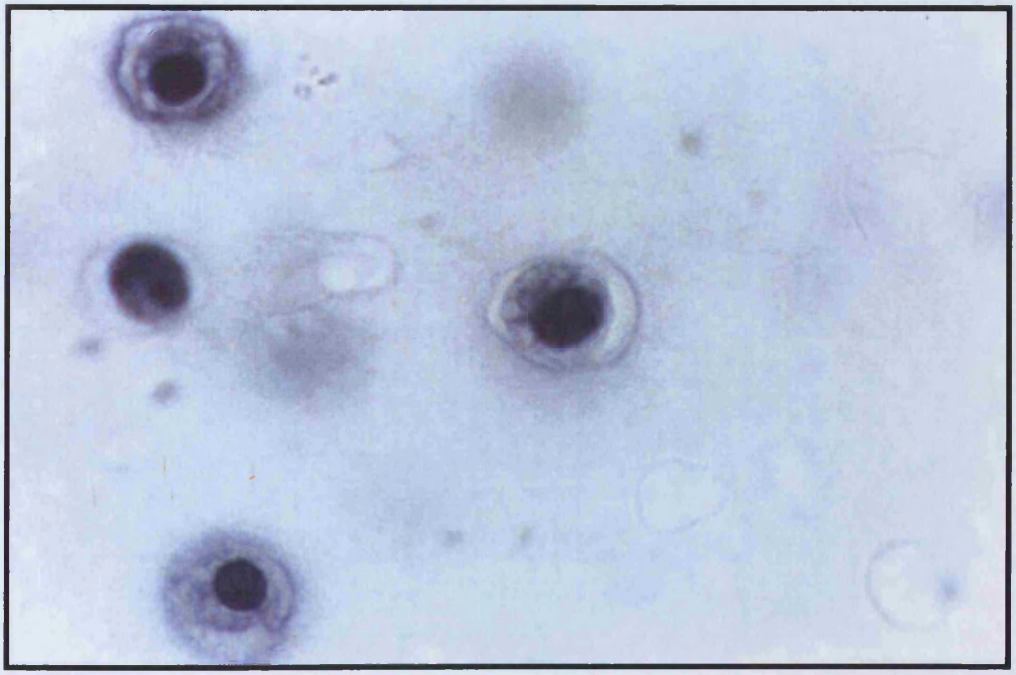


Figure 5.1.5 H& E Stain. Magnification X100.
Edge of Agarose disc construct cultured in Static culture dish for 2 days.

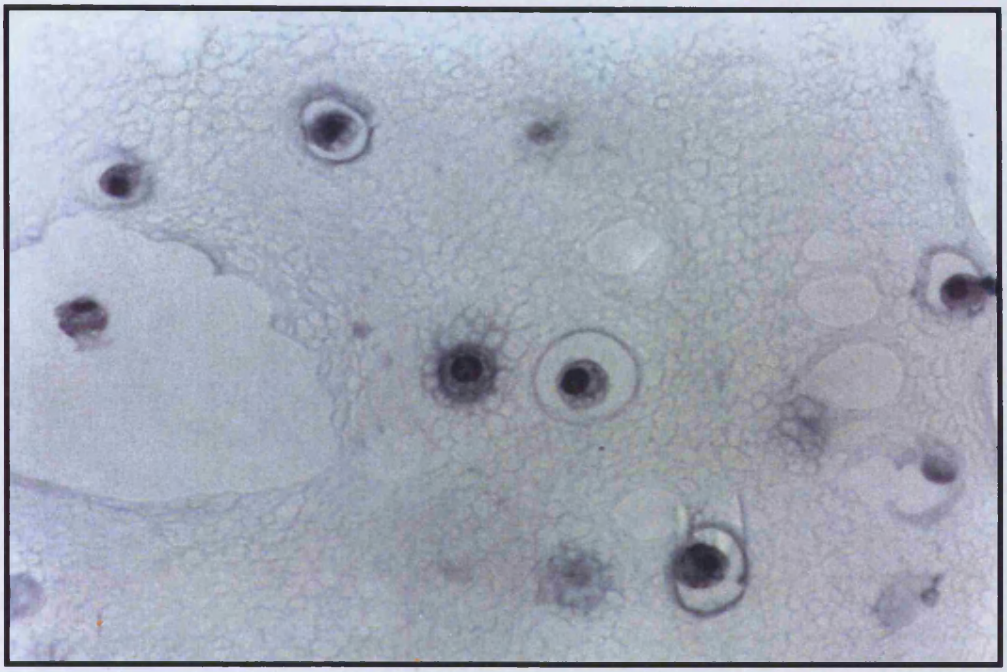


Figure 5.1.6 H&E Stain. Magnification X40.
Edge of Agarose disc construct cultured in RWV for 2 days.

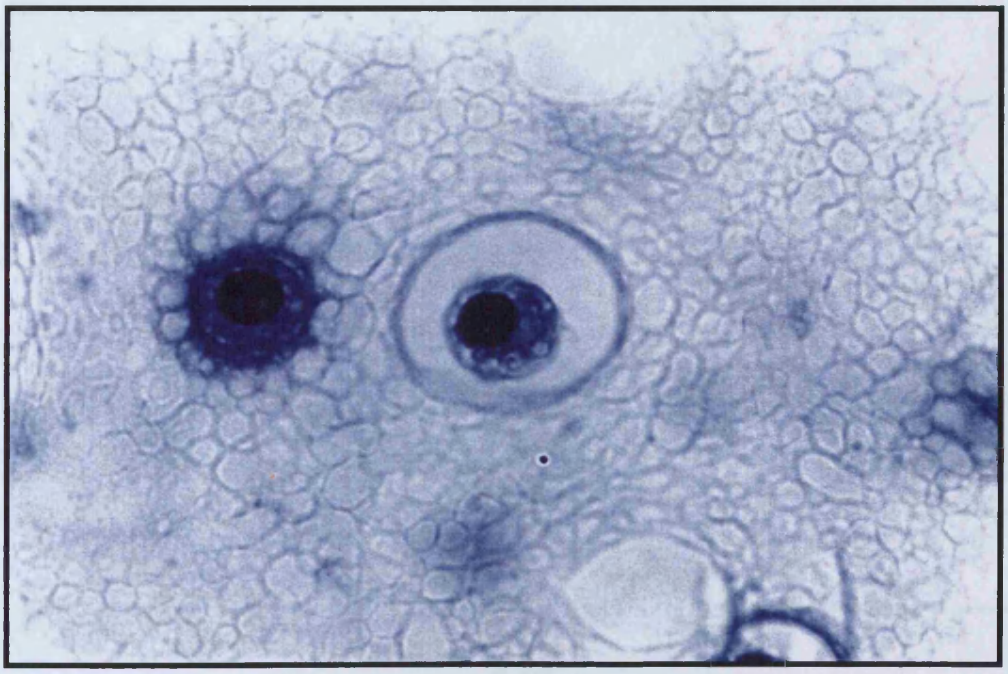


Figure 5.1.7 H&E Stain. Magnification X100.
Edge of Agarose disc construct cultured in RWV for 2 days.

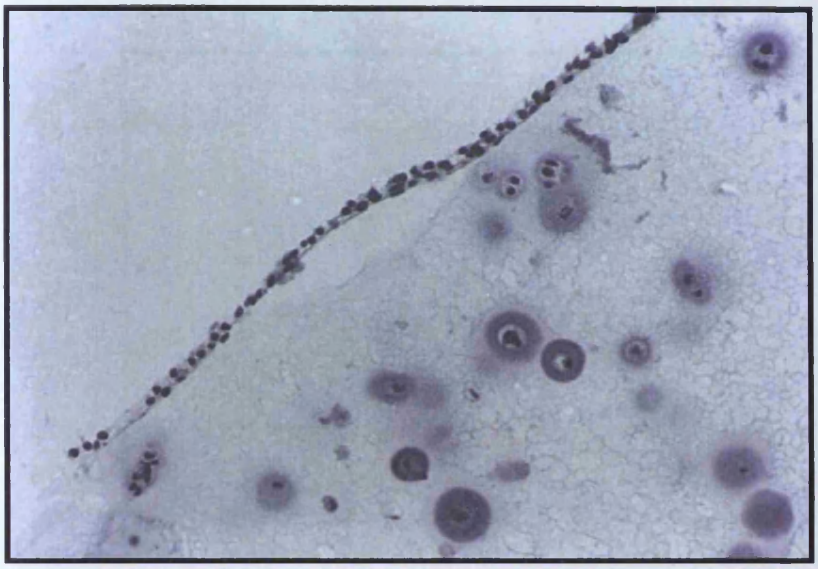


Figure 5.1.8 H&E Stain.
Magn X20.
Edge
Agarose disc
construct cultured in the
static system for 7 days.

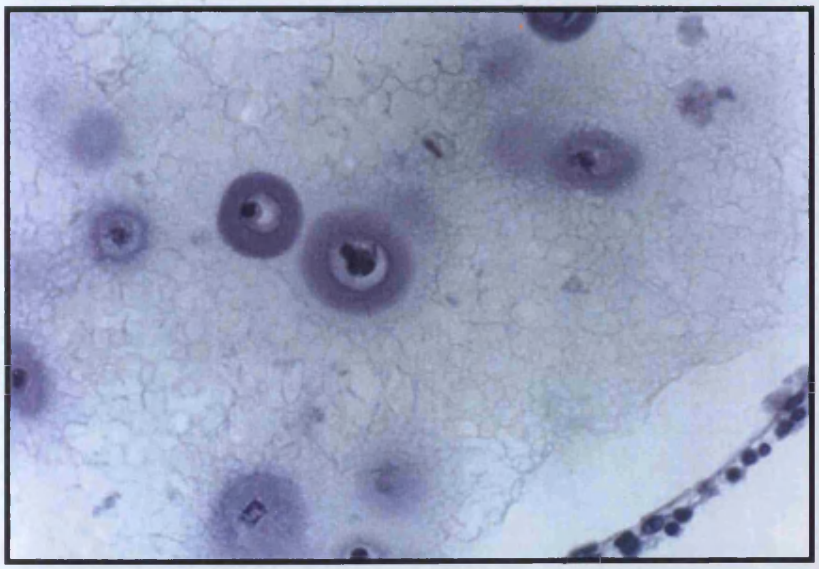


Figure 5.1.9
H&E Stain. Magn X40.
Edge
Agarose disc
construct cultured in the
static for 7 days.

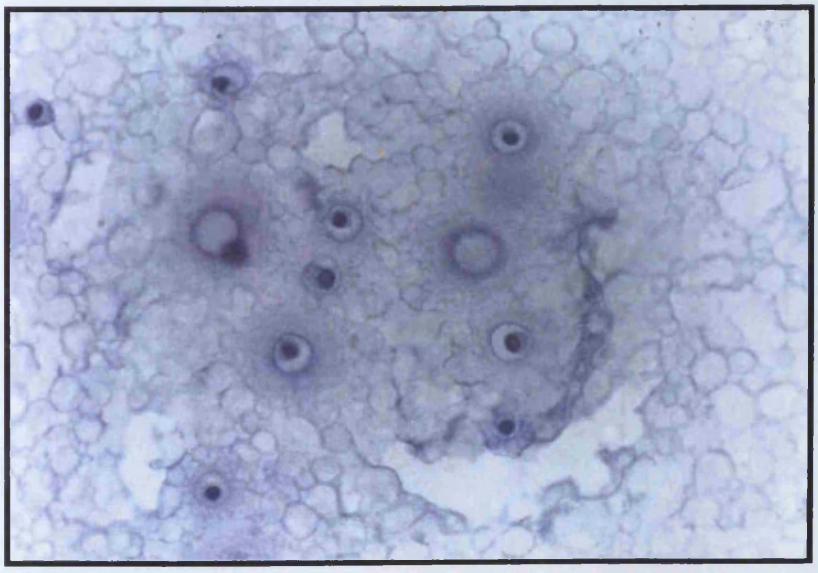


Figure 5.1.10
H&E Stain. Magn X20.
Centre
Agarose disc
Construct cultured in the
static system for 7 days.

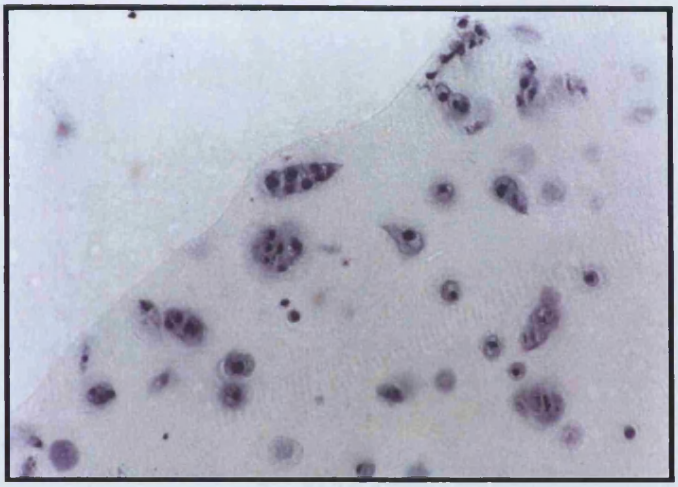


Figure 5.1.11
H&E Stain. Magn X10.
Agarose disc. Edge.
Day 7 in RWV system

Figure 5.1.12
H&E Stain. Magn X20.
Agarose disc. Edge.
Day 7 in RWV system

Figure 5.1.13
H&E Stain. Magn X20.
Agarose disc. Centre.
Day 7 RWV system

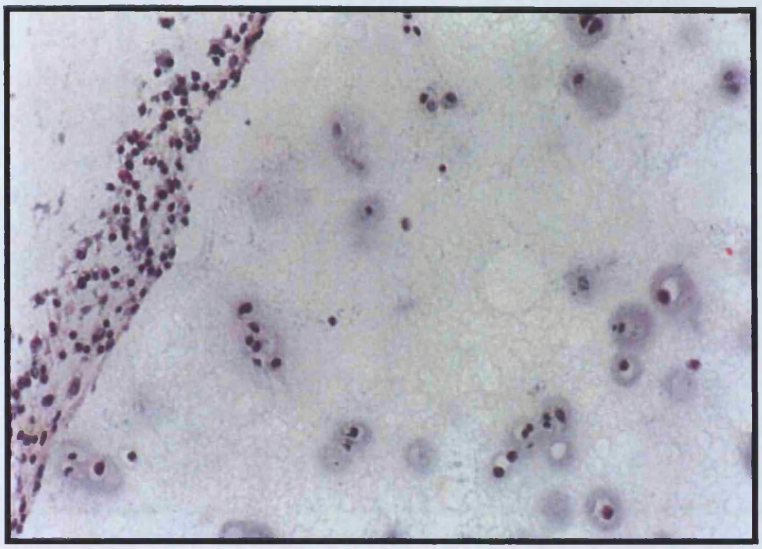


Figure 5.1.14
H&E Stain. Magn X10.
Agarose disc. Edge.
Day 12 in Static system

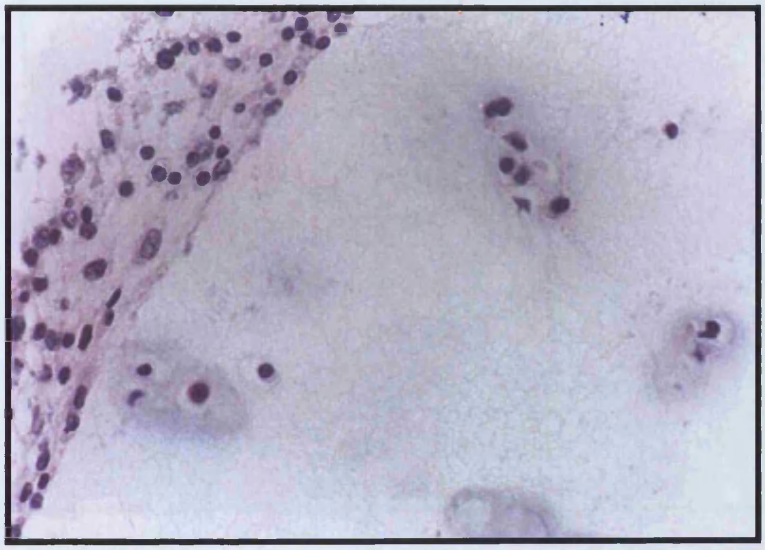


Figure 5.1.15
H&E Stain. Magn X20.
Agarose disc. Edge.
Day 12 in Static system

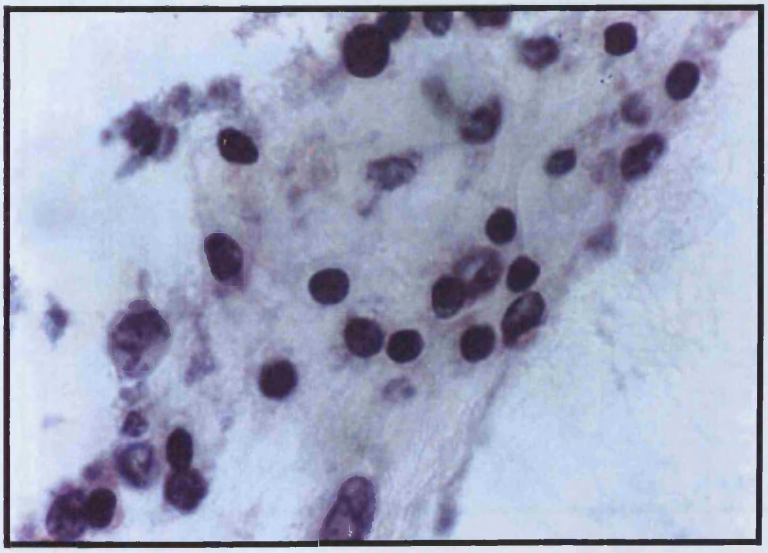


Figure 5.1.16
H&E Stain. Magn X40.
Agarose disc. Edge.
Day 12 in Static system

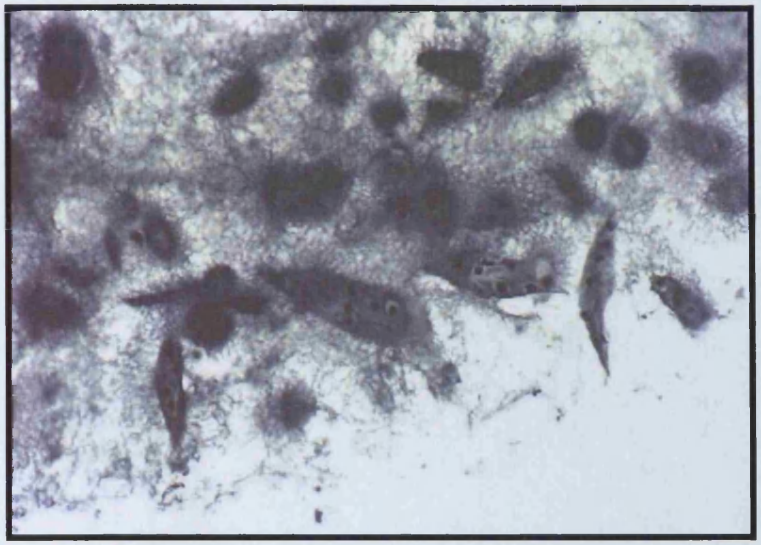


Figure 5.1.17
Tol Blue Stain. Magn X20.
Agarose disc. Edge.
Day 12 in RWV

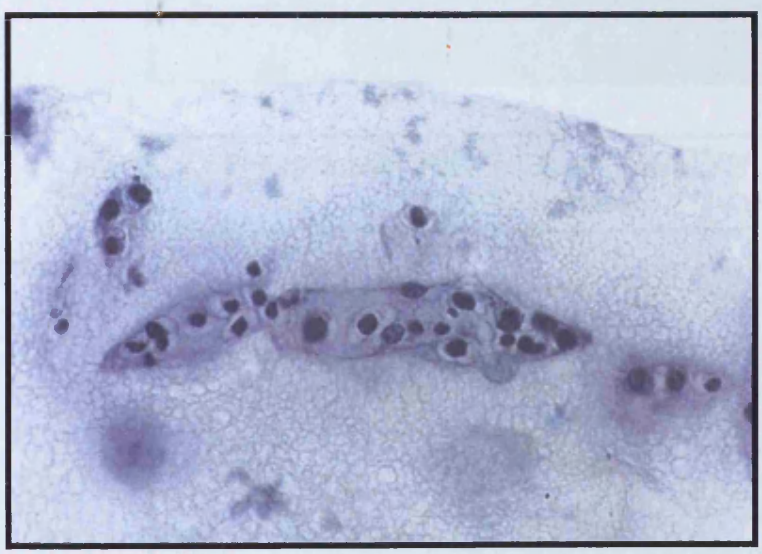


Figure 5.1.18
H&E Stain. Magn X20.
Agarose disc. Edge.
Day 12 RWV

Figure 5.1.19
H&E Stain. Magn X40.
Agarose disc. Cell Clump.
Day 12 RWV

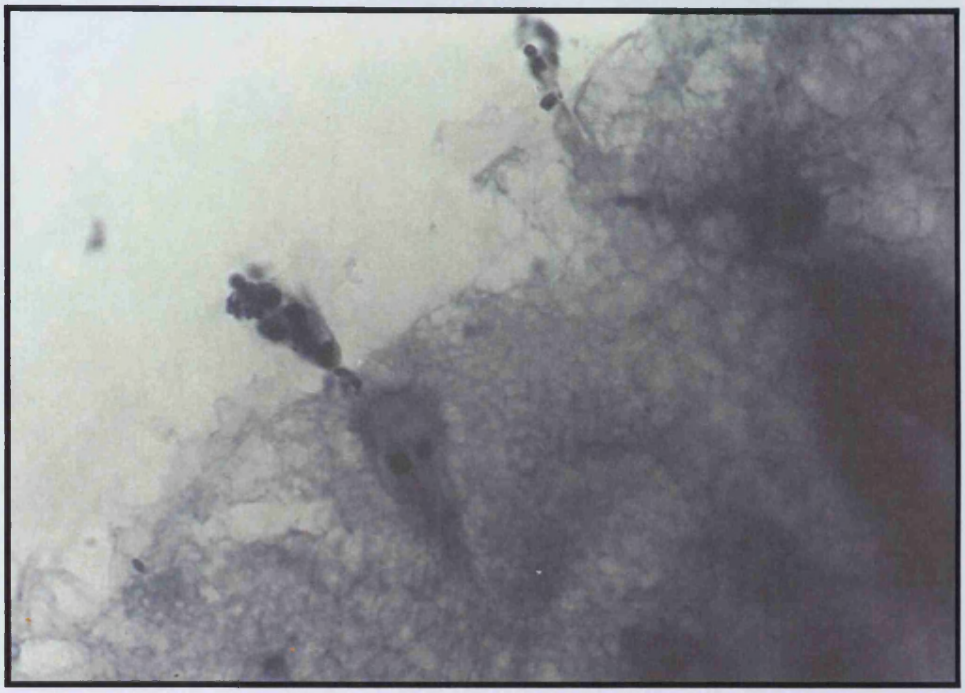


Figure 5.1.20 Tol Blue Stain. Magn X20. Edge. Agarose disc
Day 12 culture in RWV system

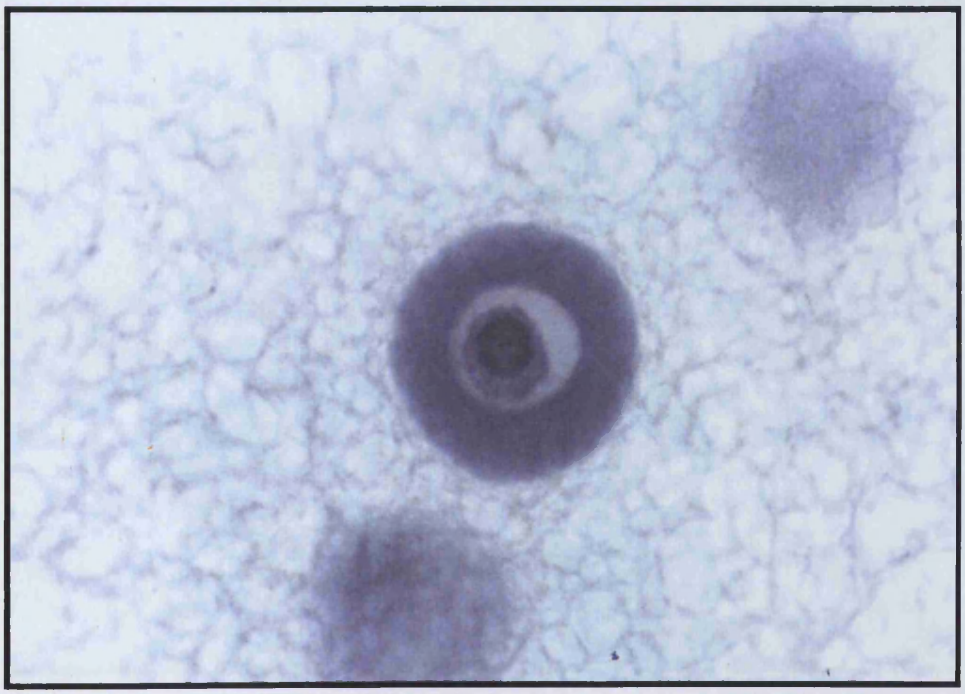


Figure 5.1.21 H&E Stain. Magn X40. Centre. Agarose disc
Day 12 culture in RWV system

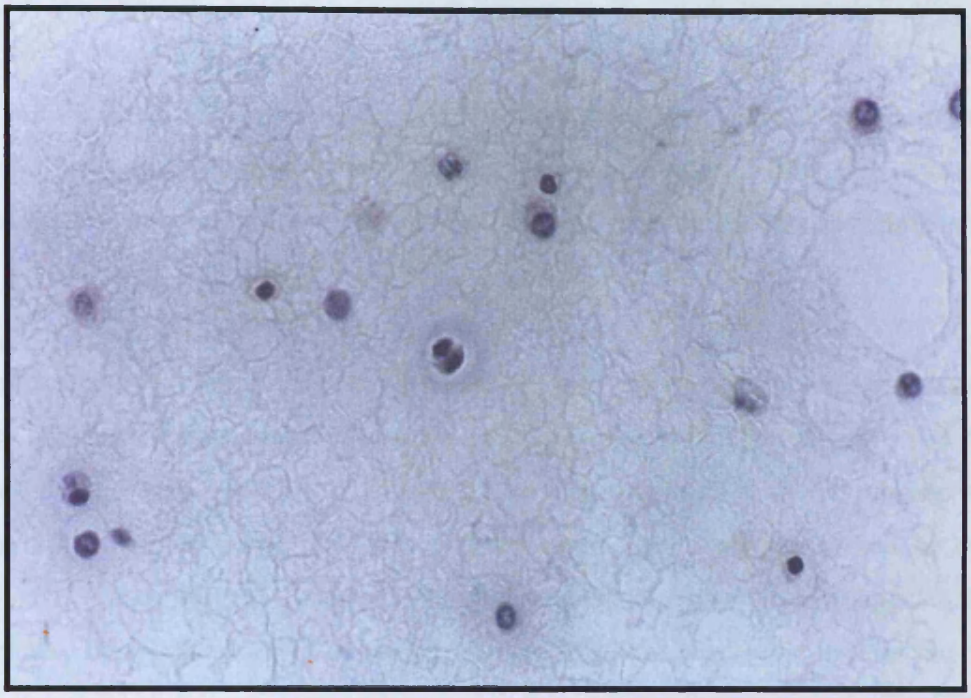


Figure 5.1.22 H&E Stain. Magn X20. Centre. Agarose disc
Day 12 culture in Static system

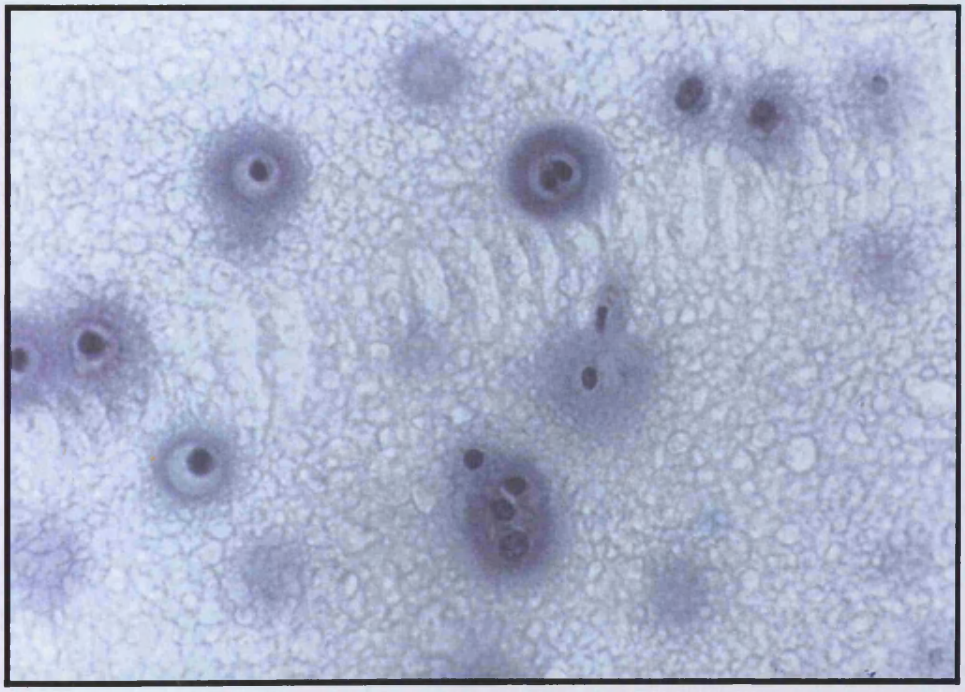


Figure 5.1.23 H&E Stain. Magn X20. Centre. Agarose disc
Day 12 culture in RWV system

5.1.3 Conclusion

The culture of chondrocytes in agarose has been successful in these experiments. The agarose not only supports the survival of chondrocytes, but also allows the differentiation of chondrocytes to form clumps of cells surrounded by H&E staining matrix.

The presence of multiple cells on the surface of the agarose disc represents a monolayer effect in which the chondrocytes grow in a manner similar to that observed on flat culture dishes. The cells appear flattened with large nuclei, minimal cytoplasm and no matrix. It is a possibility that these surface cells have dedifferentiated to become fibroblasts. The presence of these cells in only the static culture system suggests that the effect may be related to the physical contact of agarose discs to the base of the culture dish. It may be that the free chondrocytes in the medium form a monolayer of cells on the base of the culture dish and these monolayer forming cells gradually grow around the surface of the agarose discs which sit on the bottom of the culture dish. This effect does not occur in the RWV device as the discs do not make contact with the vessel walls.

The second explanation to the cause of the monolayer effect is that the cells on the surface of the construct begin to divide rapidly in the presence of plentiful nutrient medium resulting in the formation of multiple layers. The cells appear to have become fibroblastic due to the loss of their three dimensional containment. They remain attached to the surface of the agarose disc. The dynamic device has constantly moving medium and this may prevent the chondrocytes from maintaining their attachment to the disc surface.

The presence of spindle shaped clumps of chondrocytes associated with cartilage matrix at the edge of the discs in the RWV suggests that the chondrocytes experience better conditions for growth at the periphery. The cells appeared healthy and were dividing in a manner similar to that in normal articular cartilage. These type of cell clusters are not seen in the static device. Cells towards the deeper regions of the disc construct do not have the same potential to replicate and produce matrix as a result of the diffusional restraints imposed by the agarose to nutrients. In fact only the very peripherally located

cells appear to divide despite the dynamic culture conditions. The staic culture system does not demonstrate cell proliferation even at the peripheral margins within the disc.

5.2 Use of Agarose Bead constructs in the Bioreactor

From the previous experiment it was found that the chondrocytes favoured conditions at the periphery of the disc constructs. There is better diffusion of nutrients and waste products to and from cells located at the periphery and this may explain the improved growth of cells and the matrix production in these peripherally located cells. In order to further assess these effects, smaller constructs were designed. Smaller constructs would allow greater penetration of culture medium into the centre of the constructs and thereby improve the mass transfer of nutrients into the center of the constructs.

In this experiment, chondrocytes embedded in agarose beads were cultured for 14 days. The beads were approximately $1/10^{th}$ of the size as agarose discs and therefore theoretically offered greater transfer of nutrients and oxygen through the constructs. The rotating wall vessel device was compared with the static culture device.

5.2.1 Protocol

Approximately 200 agarose beads containing a chondrocyte concentration of 10 million cells per millilitre of agarose were formed using techniques described in the methods section. All procedures were performed under strict aseptic conditions and chondrocytes were harvested from freshly prepared bovine metacarpo-phalangeal joints

One hundred agarose beads were placed in a sterile culture dish with 50 mls of fresh culture medium. The majority of the beads settled at the bottom of the flat culture dish and were left undisturbed. The culture dish was then placed into the incubator. Culture medium was extracted and replaced with fresh culture medium at 48 hour intervals in both the static and dynamic culture system

For the RWV, the culture chamber was filled with 50 mls of freshly prepared culture medium and the RWV was assembled as in the previous experiment. The speed of rotation was adjusted until all agarose beads were suspended in the medium and were in a state of free fall.

Thirty beads were collected from each of the two culture systems at time points of zero days, seven days and fourteen days for analysis. Twenty beads were used for biochemical analysis and the remaining ten were used for histological analysis.

DNA and GAG analysis was performed as a batch on all samples and the results were plotted using a Microsoft excel speadsheet. Students T-Test was employed to calculate the statistical significance of any differences.

After processing and sectioning of all samples collected for histological analysis, H&E staining and Safranin-O staining was performed. Representative sections were photographed using an olympus microscope.

5.2.2 Results

5.2.2.1 Biochemistry

Table 4

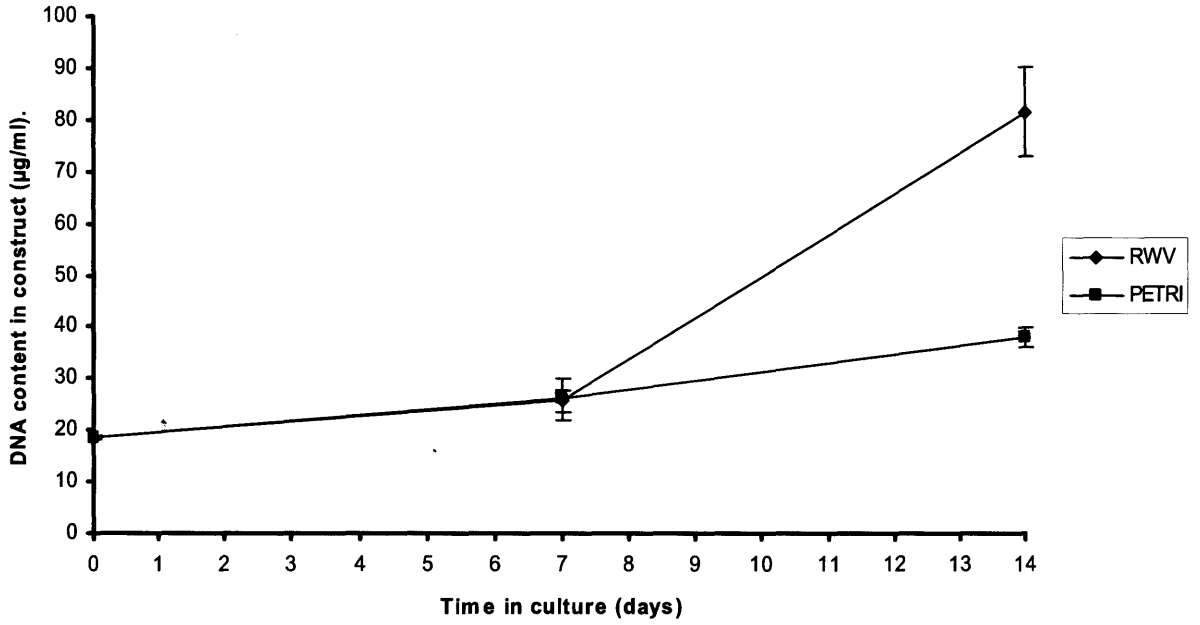
Mean DNA concentration of Agarose constructs cultured in a RWV and a Culture dish for up to 14 days

	RWV		PETRI	
Day	DNA Conc ($\mu g.ml^{-1}$)	SEM	DNA Conc ($\mu g.ml^{-1}$)	SEM
0	19	0.37	19	0.37
7	26	2	26	4
14	82	9	38	2

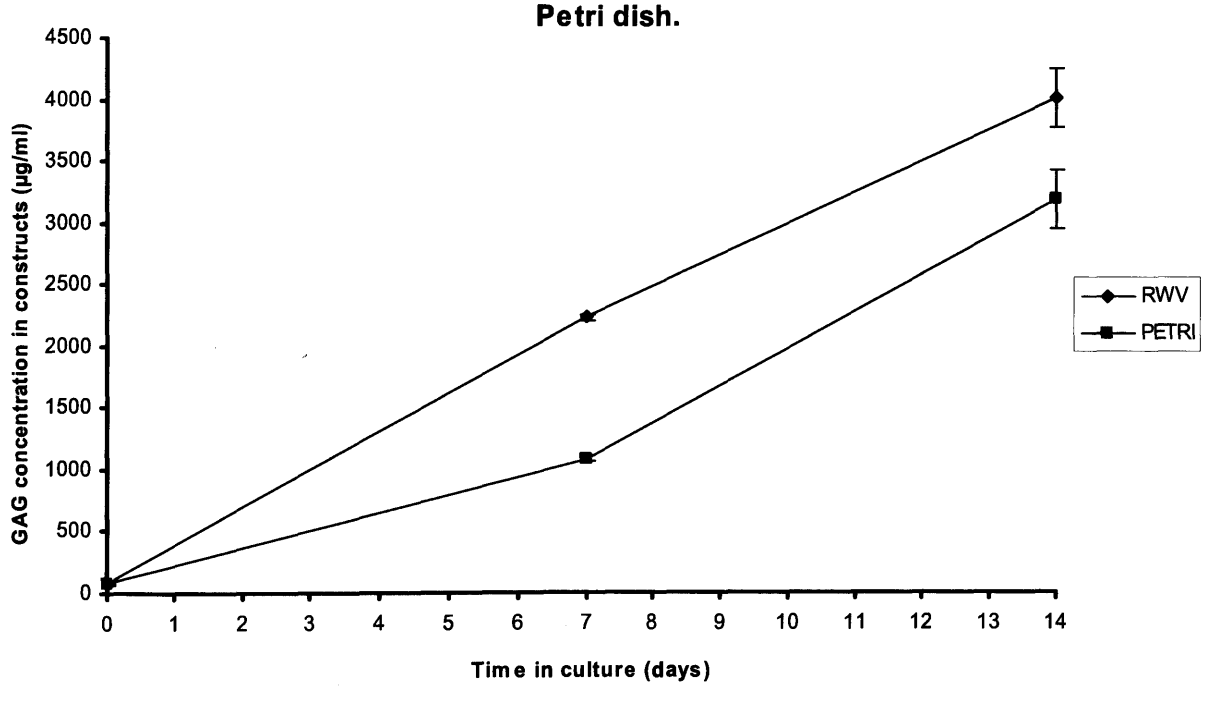
SEM = Standard Error of the Mean

Graph 8 and Table 4 show the mean DNA concentration per millilitre of agarose construct at the different time points. The initial concentration of DNA per construct was 19 $mg.ml^{-1}$ which corresponds to a cell number of 3.2 million ($19 \times 10^{-3} / 6 \times 10^{-9}$) cells per ml of construct as each chondrocyte contains approximately 6.7×10^{-9} $mg.ml^{-1}$ DNA.

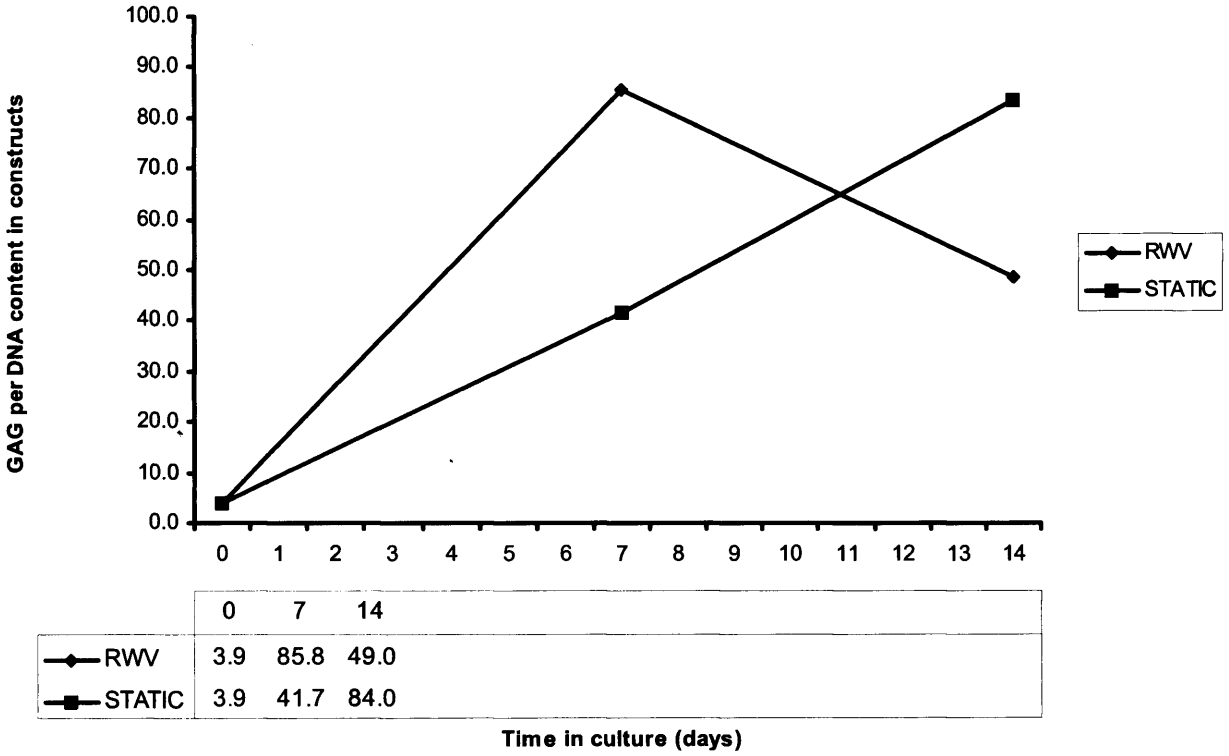
GRAPH 8. DNA content of cell seeded agarose bead constructs cultured for up to 14 days in a Dynamic Rotating Wall Vessel bioreactor and a Static Petri dish system



GRAPH 9. GAG content of cell seeded agarose bead constructs cultured for up to 14 days in a rotating wall vessel bioreactor and a Petri dish.



GRAPH 10. GAG per DNA content of cell seeded agarose bead constructs cultured for 14 days in a rotating wall vessel bioreactor and a static culture dish.



	0	7	14
RWV	3.9	85.8	49.0
STATIC	3.9	41.7	84.0

At day seven, there was a significant increase to a value of 26 $\mu g.ml^{-1}$ in the RWV and 26 $\mu g.ml^{-1}$ in the static device. This represents a 40% increase in both systems. The difference between the two culture systems was not significant ($p>0.05$)

At day 14, the constructs cultured in the RWV showed a DNA concentration of 82 $\mu g.ml^{-1}$ and the static system $\mu g.ml^{-1}$ representing an increase in DNA concentration of 4.3 fold and 2 fold respectively. The difference between the two systems was also significant ($p<0.05$ Students T-Test) at this time point.

Table 5

Mean GAG concentration of Agarose constructs cultured in a RWV and a Static culture dish for up to 14 days.

	RWV		PETRI	
Day	GAG Conc ($\mu g.ml^{-1}$)	SEM	GAG Conc ($\mu g.ml^{-1}$)	SEM
0	74	9	74	9
7	2232	23	1083	22
14	4016	237	3191	237

SEM = Standard Error of the Mean

Graph 9 and Table 5 show the mean GAG concentration per millilitre of agarose construct at the specified time points of the experiment. The GAG concentration in the bead constructs at the start of the experiment was 74 $\mu g.ml^{-1}$. By day 7, there was a large significant increase of GAG in both systems to a value of 2232 $\mu g.ml^{-1}$ in the RWV and 1083 $\mu g.ml^{-1}$ in the Static device. The GAG concentrations continue to increase and by day fourteen, levels of 4016 $\mu g.ml^{-1}$ were attained in the RWV and 3191 $\mu g.ml^{-1}$ in the static device. The differences were statistically significant at both time points and between culture systems ($p<0.05$ Students T-Test).

Graph 10 illustrates the GAG content per DNA during the culture period. Upto day 7, the ratio increases uniformly in both culture systems and implies a steady amount of

GAG production by the cells. During this period cell proliferation is minimal. By day seven the ratio of GAG/DNA has increased to 86 in the RWV and 42 in the Static system as cells continue to synthesise matrix even though there is no cell proliferation. After day 7, cell proliferation begins to occur at an increased rate in the RWV. However, despite an increase in DNA and GAG, the overall ratio of GAG/DNA falls. This implies cell proliferation rather than GAG production during this phase of culture in the RWV, whereas in the static culture system, cell proliferation and GAG production maintain a steady increase upto day 14. The overall DNA and GAG concentration in this system is far higher than the agarose disc system.

5.2.2.2 Histology

Figures 3.4.1, 3.4.2 and 3.4.3 are control specimens displaying the appearance of a normal section of bovine articular cartilage. Figure 3.4.1 is a Safranin-O stain. There is intense staining of proteoglycans in the extracellular matrix. There is significant staining throughout the whole specimen. Figure 3.4.2 is a haematoxylin and eosin stain. The appearance of cells and cell clusters can be identified. The spindle configuration of the chondrons is well displayed. On occassions there are clusters composed of 4 or more cells with surrounding matrix. *Figure 3.4.3* is a collagen type II immunolabel. Collagen type II is detected throughout the cartilage section and greater amounts appear to occur towards the periphery. Collagen type I immunolabelling was also performed. There was no identifiable levels of this detected in the normal articular cartilage.

Figure 5.2.1 shows the peripheral margins of an agarose bead construct at day zero time point. The chondrocytes are well distributed within the agarose. There are no cell clusters and no evidence of extracellular matrix (ECM). *Figure 5..2.4* is a central region of an agarose bead at a higher magnification showing individual chondrocytes composed of a nucleus and cytoplasm. The chondrocytes appear rounded and healthy. They are well dispersed within the agarose

Figures 5.2.3, 5.2.4 and 5.2.5 show chondrocytes in agarose beads after 7 days in static culture. There is an area of ECM surrounding each chondrocyte. *Figure 5.2.4* shows cell clusters on the surface from the agarose construct. Chondrocytes cultured in static conditions seem to have a tendency to attach to surfaces and then multiply and it may be this phenomenan that is responsible for the cell clusters at the agarose bead surface. It is

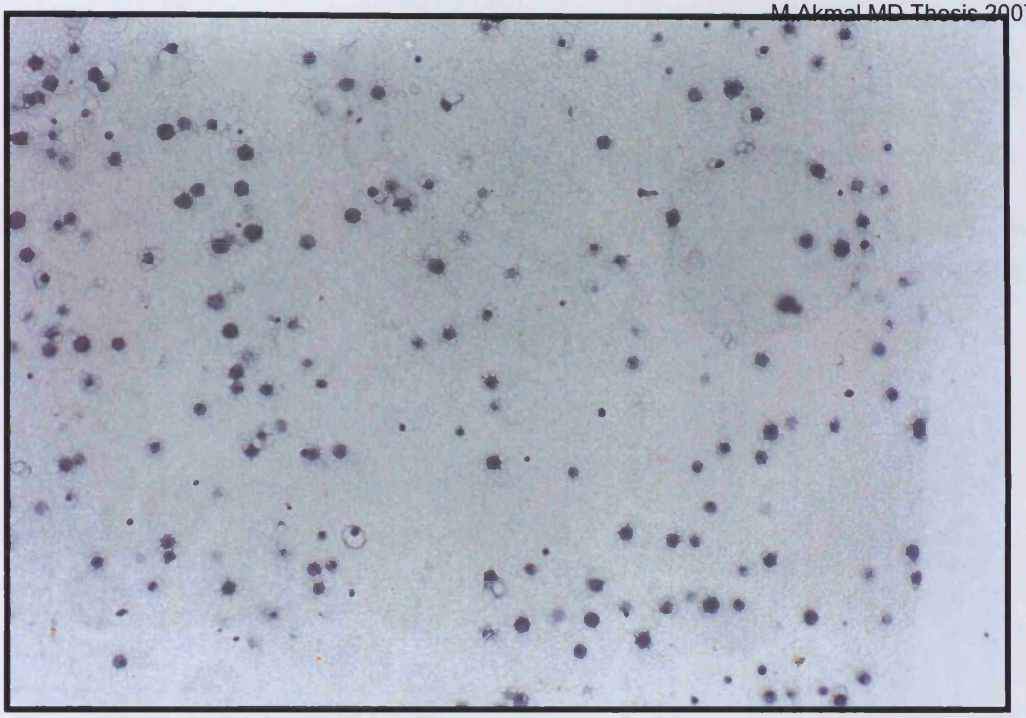


Figure 5.2.1 H&E Stain. Magn X10. Edge. Agarose bead
Day zero centre of agarose disc construct containing chondrocytes

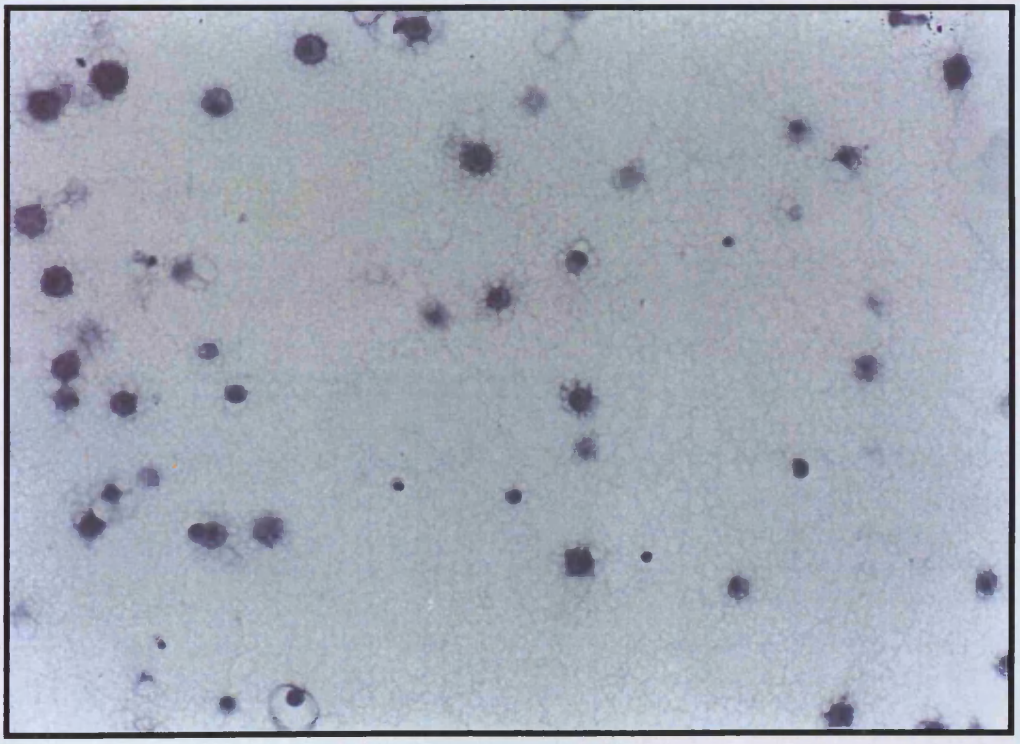


Figure 5.2.2 H&E Stain. Magn X20. Centre. Agarose bead
Day zero centre of agarose disc construct containing chondrocytes

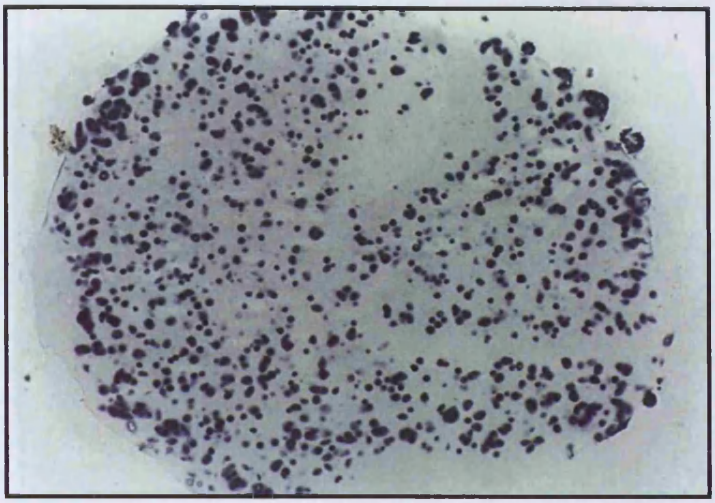


Figure 5.2.3
H&E Stain. Magn X4.
Agarose bead construct cultured in the Static system for 7 days.

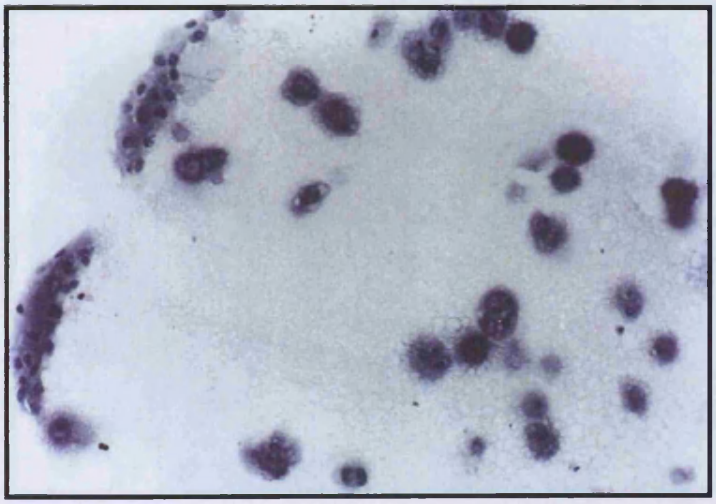


Figure 5.2.4
H&E Stain. Magn X20.
Edge of Agarose bead construct cultured in the Static system for 7 days.

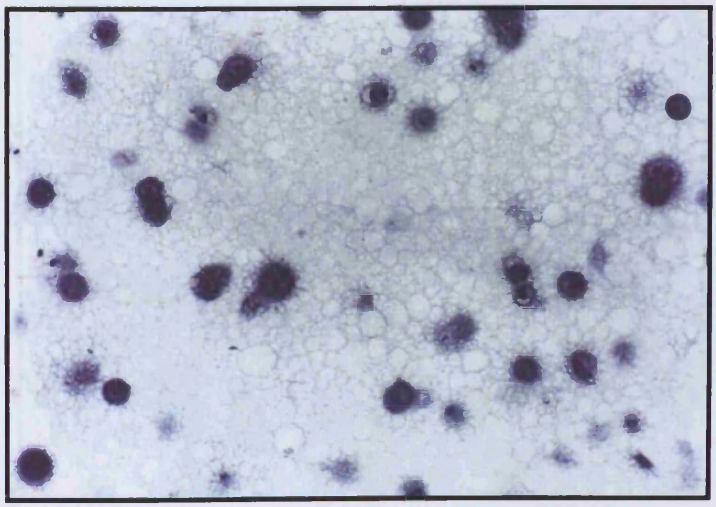


Figure 5.2.5
H&E Stain. Magn X20.
Centre of Agarose bead construct cultured in the Static system for 7 days.

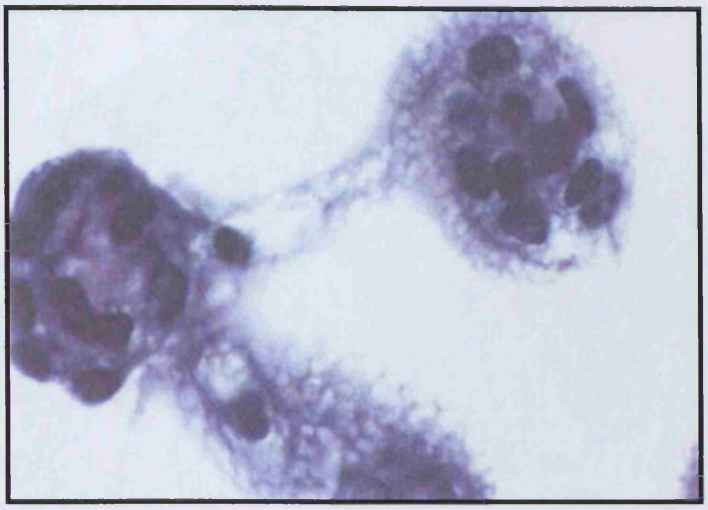


Figure 5.2.6
H&E Stain. Magn X100.
Cell clump in Agarose bead construct cultured in the RWV system for 7 days.

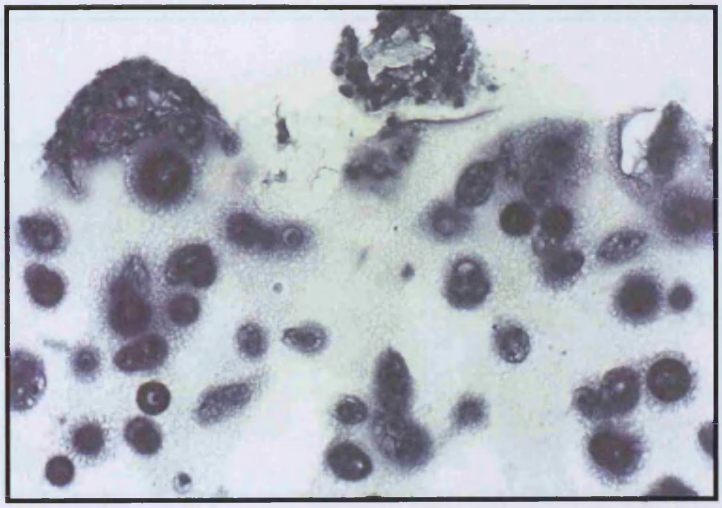


Figure 5.2.7
H&E Stain. Magn X20.
Edge of Agarose bead construct cultured in the RWV system for 7 days.

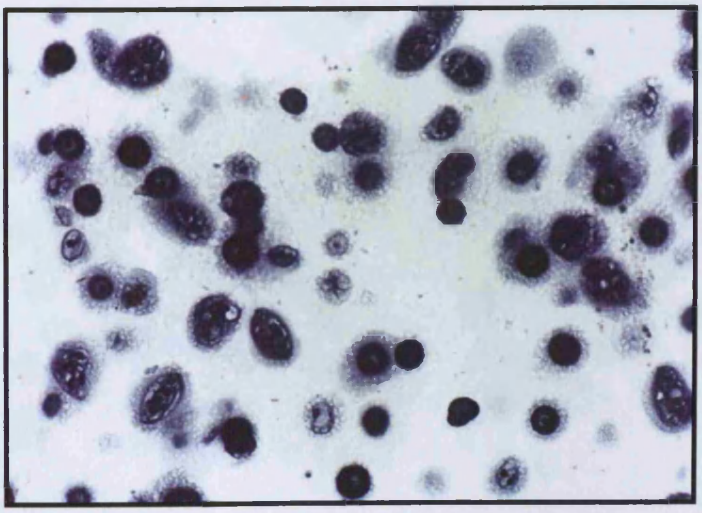


Figure 5.2.8
H&E Stain. Magn X20.
Centre of Agarose bead construct cultured in the RWV system for 7 days.

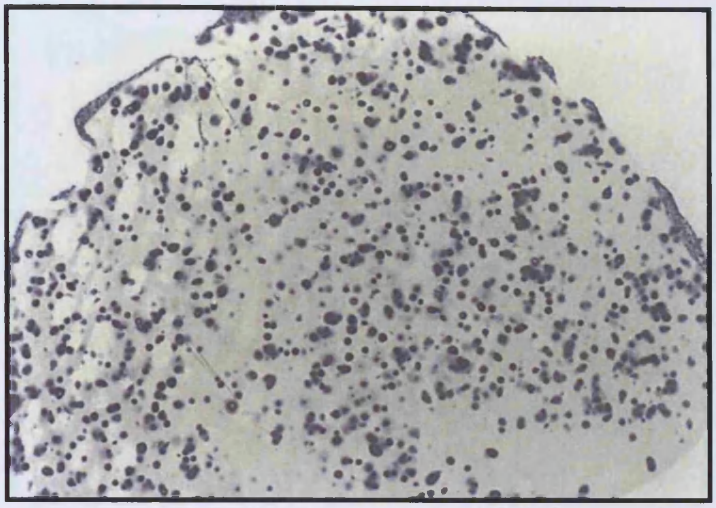


Figure 5.2.9
H&E Stain. Magn X4.
Agarose bead construct cultured in the Static system for 14 days.

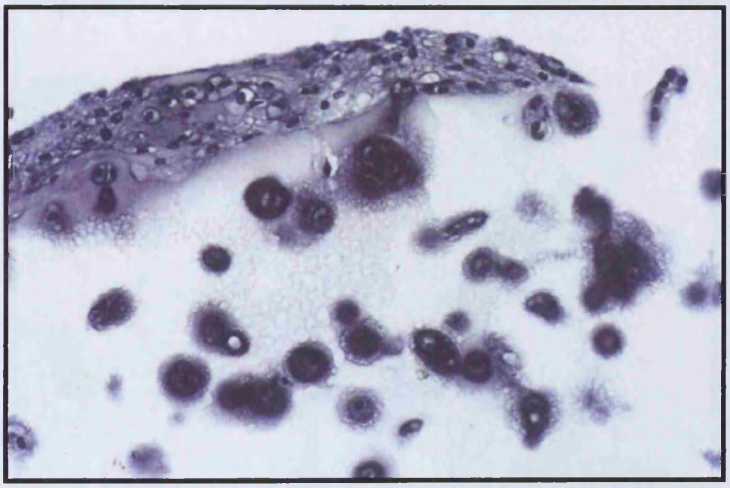


Figure 5.2.10
H&E Stain. Magn X20.
Edge of Agarose bead construct cultured in the Static system for 14 days.

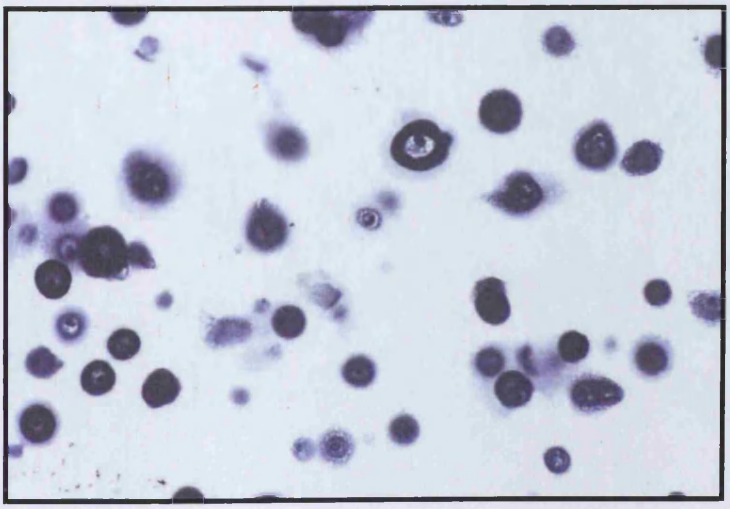


Figure 5.2.11
H&E Stain. Magn X20.
Centre of Agarose bead construct cultured in the Static system for 14 days.

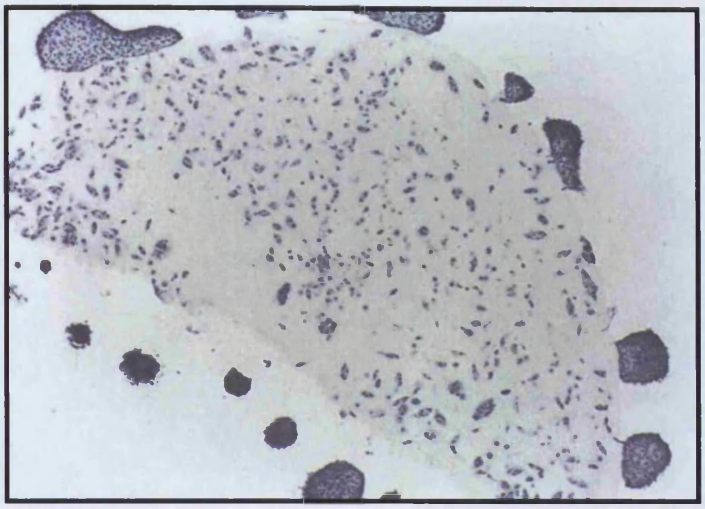


Figure 5.2.12
H&E Stain. Magn X4.
Agarose bead construct cultured in the RWV system for 14 days.

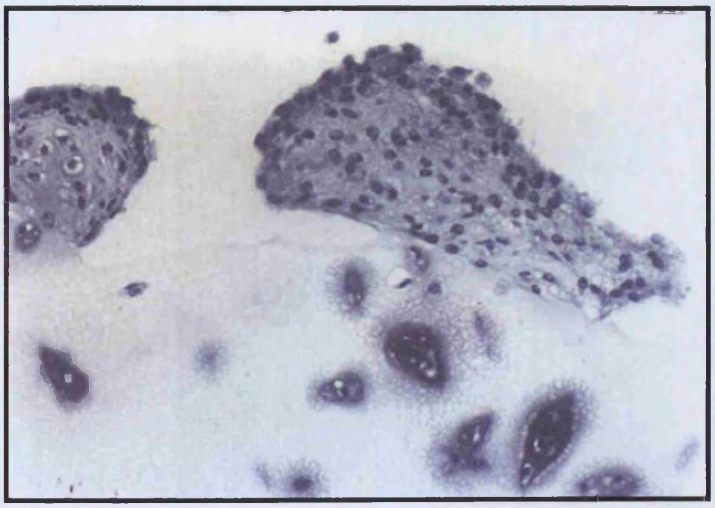


Figure 5.2.13
H&E Stain. Magn X20.
Edge of Agarose bead construct cultured in the RWV system for 14 days.

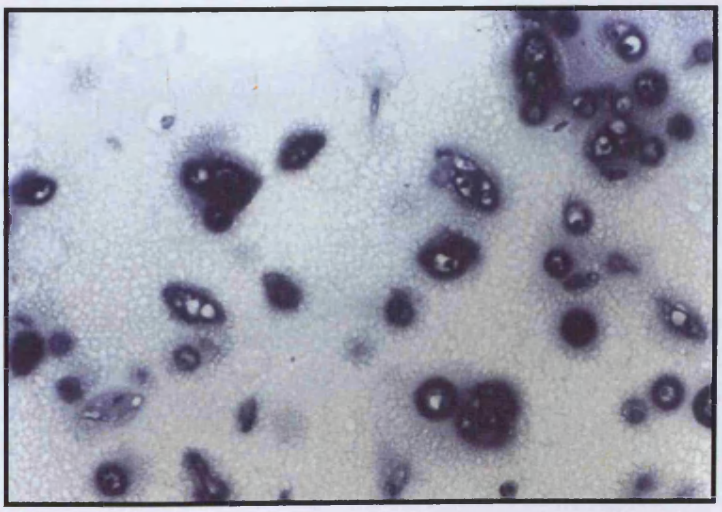


Figure 5.2.14
H&E Stain. Magn X20.
Centre of Agarose bead construct cultured in the RWV system for 14 days.

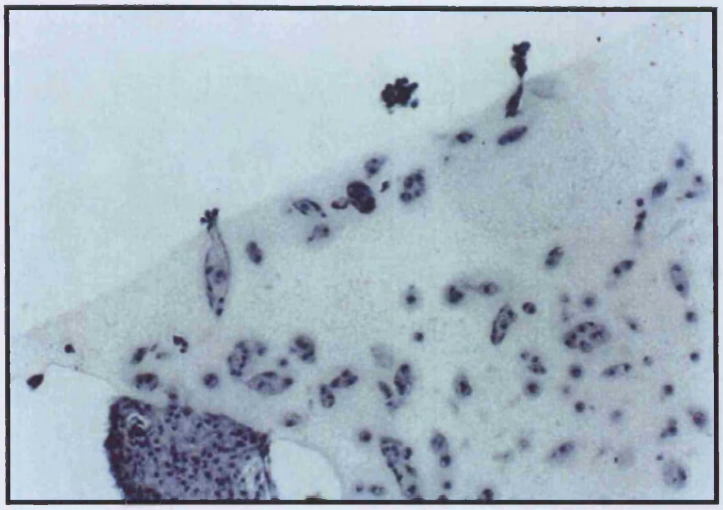


Figure 5.2.15
H&E Stain. Magn X10.
Agarose bead
construct cultured in the
RWV system for 14 days.

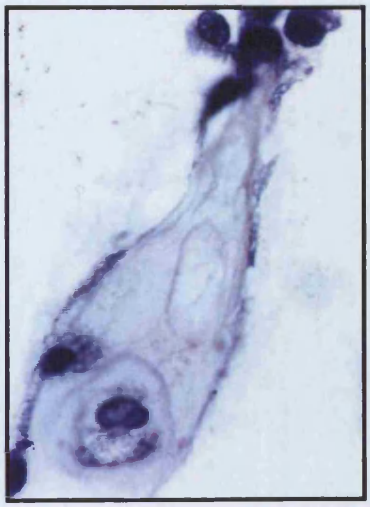


Figure 5.2.16
H&E Stain. Magn X40.
Cells adjacent to surface of
Agarose bead
construct cultured in the
RWV system for 14 days.

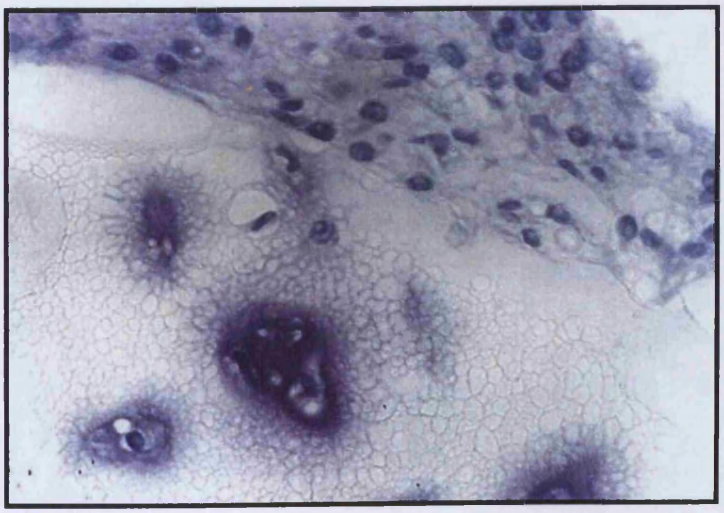


Figure 5.2.17
H&E Stain. Magn X40.
Comparison of cells within
the construct as compared
to those outside the
Agarose bead
construct cultured in the
RWV system for 14 days.

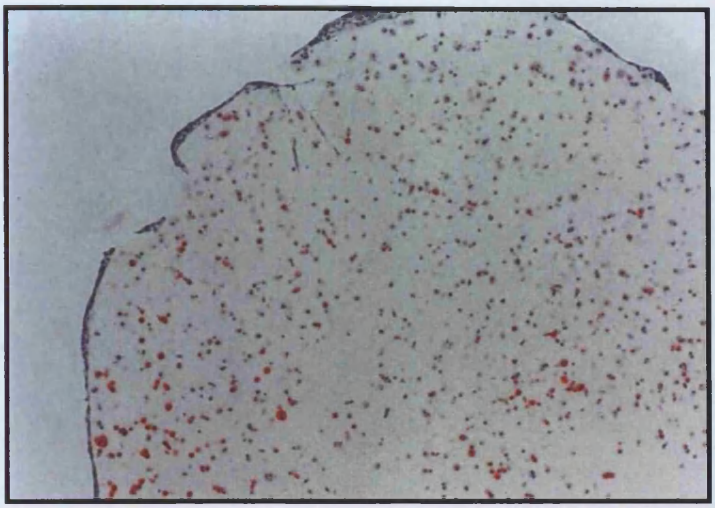


Figure 5.2.18
Saf-O Stain. Magn X4.
Agarose bead construct cultured in the Static system for 14 days.

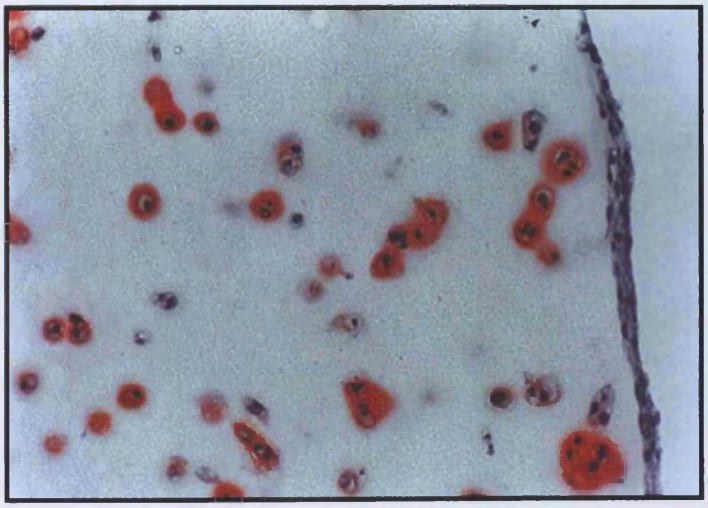


Figure 5.2.19
Saf-O Stain. Magn X20.
Edge of Agarose bead construct cultured in the Static system for 14 days.

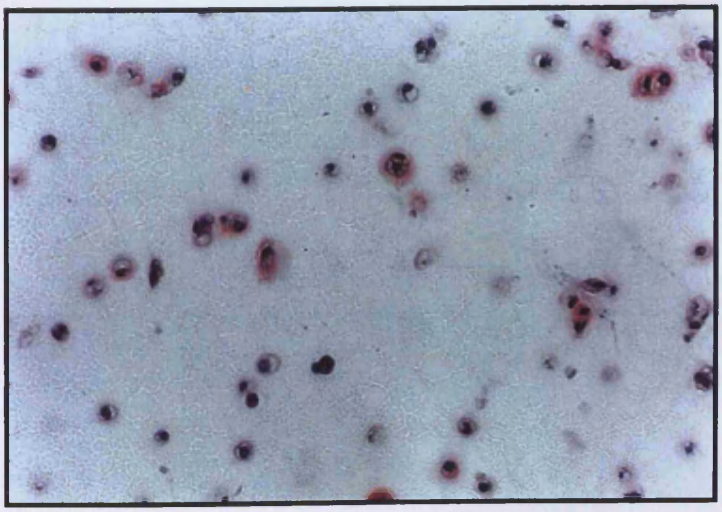


Figure 5.2.20
SAf-O Stain. Magn X20.
Centre of Agarose bead construct cultured in the Static system for 14 days.

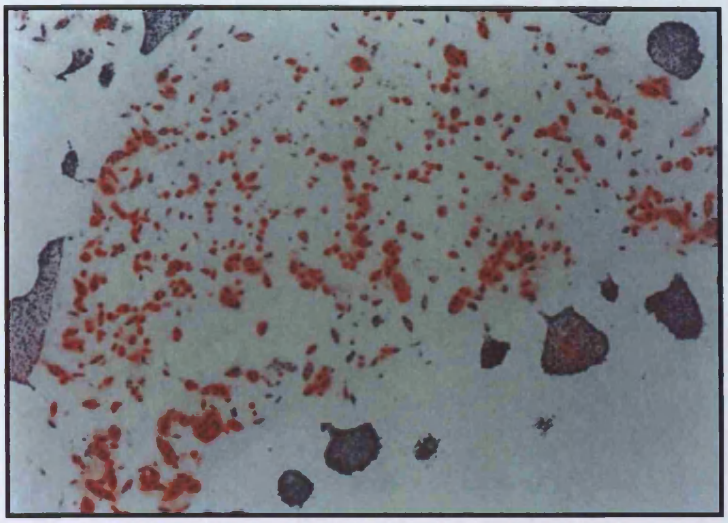


Figure 5.2.21
H&E Stain. Magn X4.
Agarose bead construct cultured in the RWV system for 14 days.

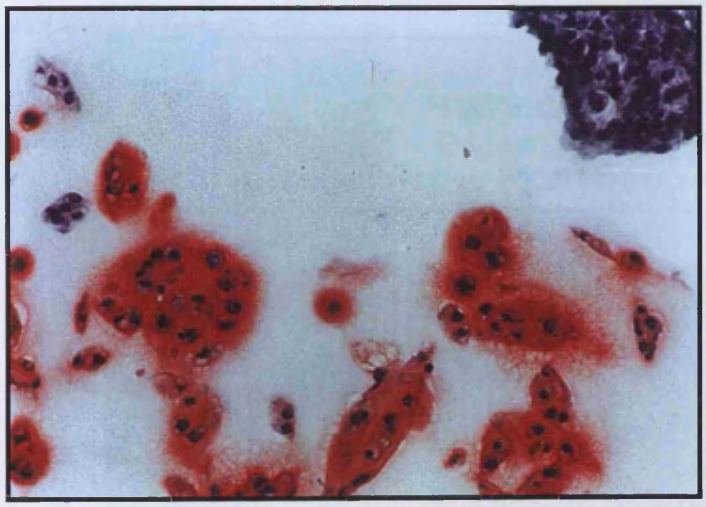


Figure 5.2.22
H&E Stain. Magn X20.
Edge of Agarose bead construct cultured in the RWV system for 14 days.

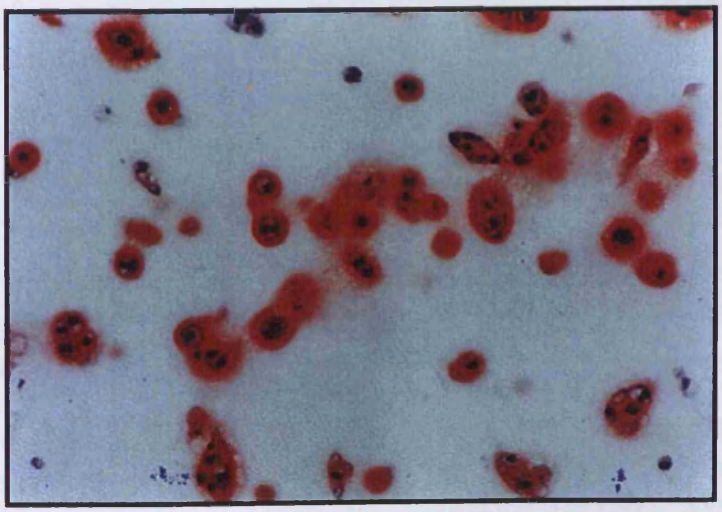


Figure 5.2.23
H&E Stain. Magn X20.
Centre of Agarose bead construct cultured in the RWV system for 14 days.

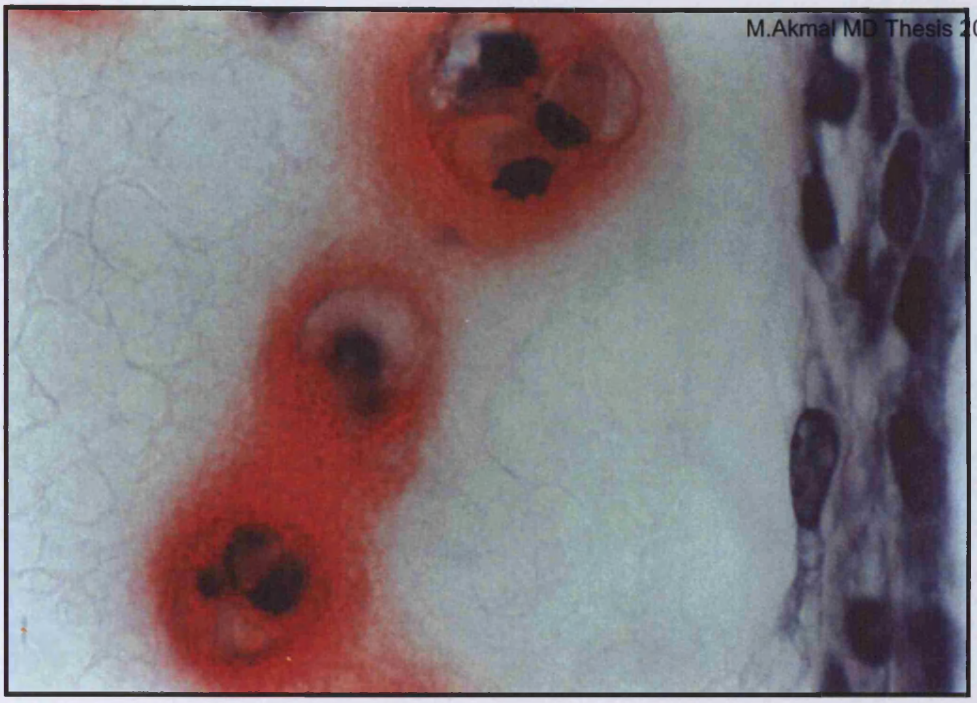


Figure 5.2.24
H&E Stain. Magn X100.
Edge of Agarose bead
construct cultured in the Static system for 14 days.

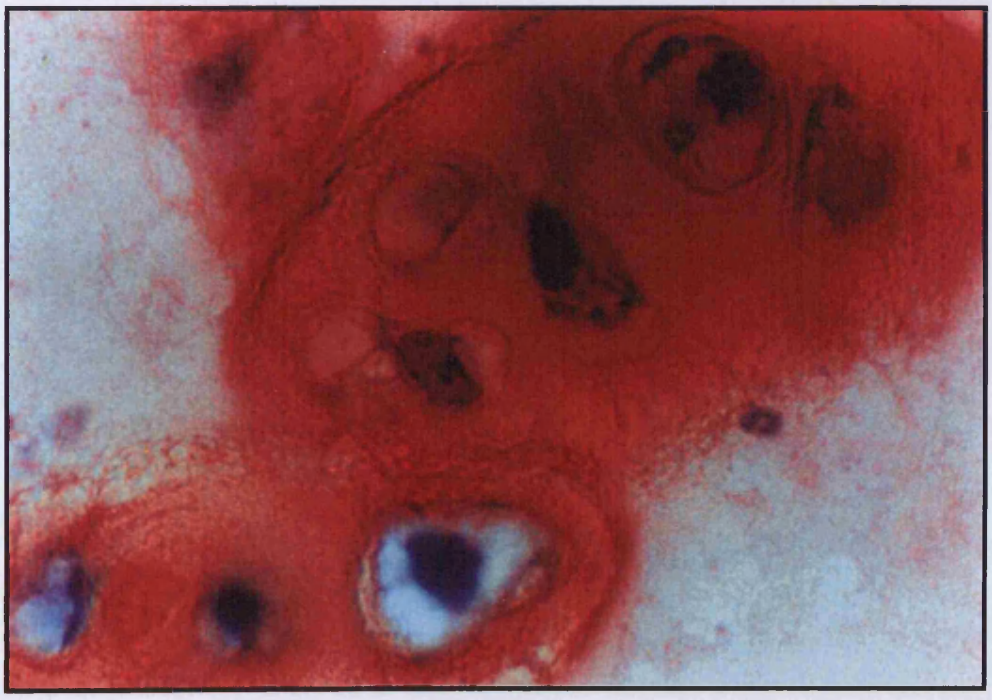


Figure 5.2.25
H&E Stain. Magn X100.
Edge of Agarose bead
construct cultured in the RWV system for 14 days.

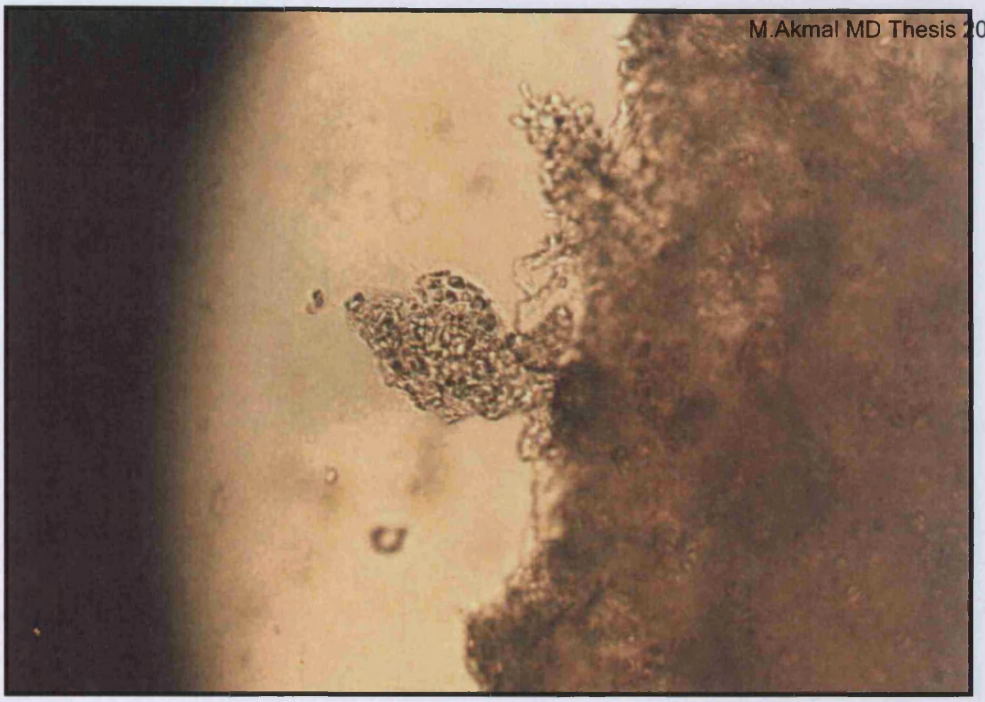


Figure 5.2.26 Phase Contrast Microscopy. Magn X20.
Edge of Agarose bead
construct cultured in the RWV system for 7 days.

Figure 5.2.27 Phase Contrast Microscopy. Magn X20.
Edge of Agarose bead
construct cultured in the RWV system for 14 days.

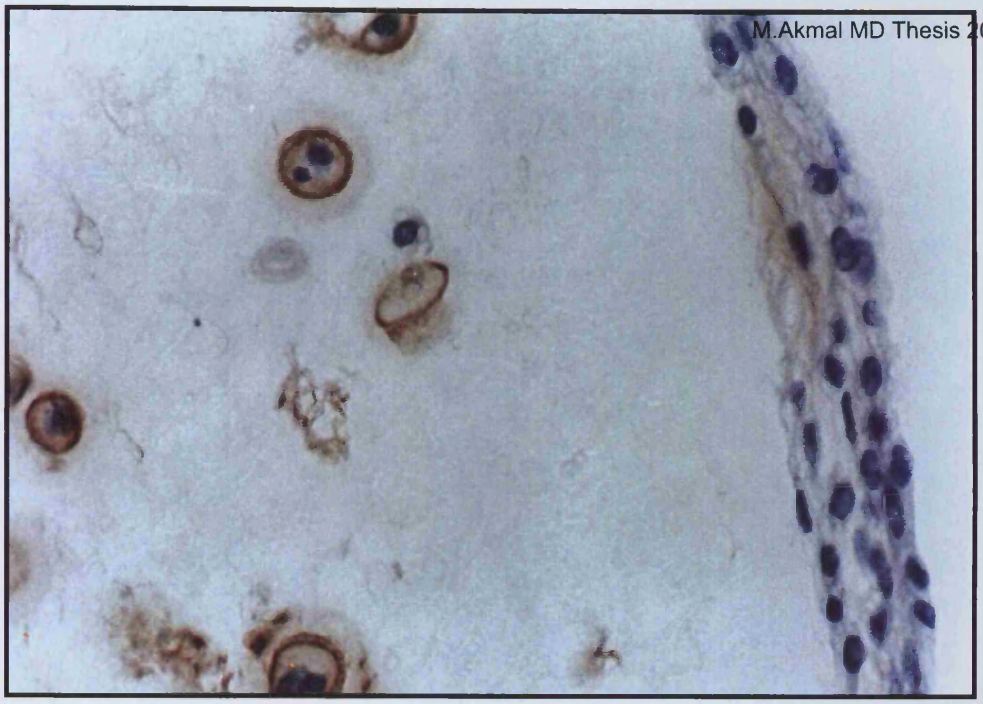


Figure 5.2.28 Collagen Type II Immunolabelling. Magn X20.
Edge of Agarose bead
construct cultured in the Static system for 14 days.

Figure 5.2.29 Collagen type II immunolabelling. Magn X20.
Edge of Agarose bead
construct cultured in the RWV system for 14 days.

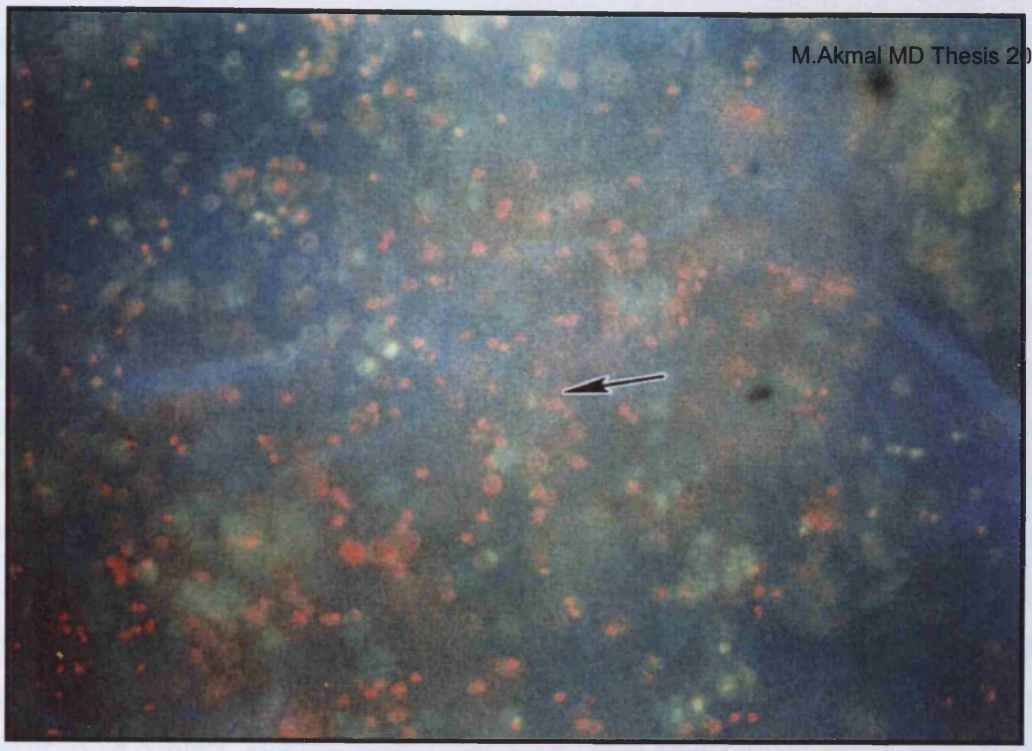


Figure 5.2.30 Calcein/Homodimer Fluorescent Stain.
Magn X10. Agarose bead
Construct cultured in the static system for 14 days

Figure 5.2.31 Calcein/Homodimer Fluorescent Stain.
Magn X10. Agarose bead
Construct cultured in the RWV for 14 days

of interest to note that even at the centre of the agarose beads there is matrix staining and the cells appear viable. There is an obvious contrast between the central areas and the periphery of the beads.

Figures 5.2.6, 5.2.7 and 5.2.8 show chondrocytes after 7 days in dynamic culture. The differences between the two systems are obvious, there is more matrix surrounding the cells than in the case of the static system. The matrix appears to have 2 zones, an inner less intensely staining zone which corresponds to the pericellular matrix and an outer more intensely staining zone corresponding to the territorial matrix. There is ample evidence of cell division and in particular there is much more matrix staining throughout the bead. The peripherally located cells appear to be dividing rapidly with a greater amount of matrix. The cell clusters are again present but they take up a different configuration as compared to the static device. They form spherical clusters with only short areas of contact with the agarose bead. The high power image figure 5.2.6 displays the nature of these cells and demonstrates the "cord like" attachment the cell cluster maintains with the original bead construct.

Figures 5.2.9, 5.2.10 and 5.2.11 show constructs cultured in the static culture dish at day 14 at different levels of magnification. The monolayer of cells has become multilayered and some of the cells within the agarose construct have now divided to form cell clumps. The cell proliferation has been dramatic and there are clusters composed of more than 100 cells per cluster at the surface. There is also staining of the matrix that is associated with these superficial cell clusters. It is also noticeable that the cells located at the peripheral regions within the agarose constructs are dividing more rapidly that the centrally located cells with a greater amount of matrix staining. There are however some cells within the central regions that do not appear viable in the cytoplasm has diminished, the nucleus has become diminished in size and become dense with no associated matrix.

Figures 5.2.12, 5.2.13, 5.2.14 show constructs cultured in the RWV at day 14 at different levels of magnification. The situation is significantly different from the statically cultured constructs. There are large spindle shaped cell clumps which often consist of 10 or more cells. The three layers of matrix are identifiable and in some places the inter-territorial matrix of neighbouring clumps has combined. There are large spherical clumps of cells attached to the surface. There does not appear to be any

particular direction in which the clumps form and they are composed of hundreds of cells. It is likely that some processing artifact has led to the appearance of separation of the clumps from the agarose surface.

Figures 5.2.15 and 5.2.16 demonstrate a similar appearance to agarose discs. As in the case of agarose discs, chondrocytes appear to become extruded from the agarose bead construct and appear to clump at the surface. They remain attached to the agarose through a stalk of matrix and typically they do not stain for matrix until a large aggregate of cells has formed. *Figure 5.2.17* shows the staining of matrix within the very large cluster of cells at the agarose bead surface.

Safranin-O staining for proteoglycans improves the contrast between proteoglycans and agarose. *Figure 5.2.18* is a low magnification image showing positive safranin-O staining around cells in the static culture. At a higher magnification, *figures 5.2.19 and 5.2.20*, the intensity of matrix staining is well displayed. Around the peripheral regions of the construct there is clearly more intense staining then around the central regions. This is consistent with the H&E staining results in previous figures.

The RWV has dramatic effects on Safranin-O staining intensity and *figures 5.2.21, 5..2.22 and 5.2.23* highlight the considerable increase in intensity of staining in the dynamic culture system. Despite cell proliferation with cell clusters composed of more than 12 cells, there is still intense matrix staining throughout the construct. There does however tend to be lower intensity staining at the centre of the constructs as compared to the periphery.

The high magnification images, *figures 5.2.24 and 5.2.25,* demonstrate the clear demarcation between the intense matrix staining and the non-stained agarose gel. There is a well demarcated border between the chondrocytes and matrix. The nuclei are distinguishable and there is good evidence for mitosis.

Figures *5.2.26 and 5.2.27* are contrast microscopy images of agarose beads at day 14 culture. These specimens were not stained and were fresh specimens. The previous phenomena of cell extrusion and cell clump formation seen in agarose discs and beads can also been seen in these images. This event of cell clump formation around the

periphery of the constructs is therefore a real phenomenon and does not represent a processing artefact.

Immunolabelling for collagen subtypes is one of the most accurate and definitive methods of confirming the nature of regenerated tissue. *Figures 5.2.28 and 5.2.29* are collagen type II immunolabelled constructs at 14 days of culture. *Figure 5.2.28* is a statically cultured specimen which demonstrates the presence of staining around the cells in the construct. It is of high relevance that there is no staining of collagen type II in the multilayered cells around the surface whereas cells embedded within the matrix show intense collagen type II staining. *Figure 5.2.29* is a specimen cultured in the RWV and demonstrates significantly greater intensity of staining and a much more organised distribution of collagen type II staining. There is intense collagen type II pericellular staining and the chondrocyte clumps are clearly visible. The cells appear healthy and the agarose appears densely packed with matrix. The typical spindle configuration is adopted by all the cell clumps. There is no clear alignment of the cell clumps and they appear to be randomly aligned.

The assessment of cell viability is important in determining the characteristics of any scaffold material. The Calcein and homodimer stain that was used for cell viability assessment distinguishes between live and dead cells. The calcein fluorescent stain is taken up by living cells and appears green under fluorescent light microscopy. The Homodimer stain is taken up by dead cells and appears red under fluorescent light microscopy. *Figure 5.2.30* displays an agarose bead construct extracted from the static culture experiment at day 14 and immediately stained with the live/dead cell stain and examined by fluorescent microscopy. There is a clear demonstration of live cells (green) at the peripheral regions of the construct and less viable cells (red) towards the centre and deeper areas of the construct. Cell viability dramatically decreases in relation to the distance from the construct surface. *Figure 5.2.31* displays an agarose bead extracted from the RWV culture at the same time point as above. In this case the demarcation between live cell and dead cell staining is not so clear. There is still however an appearance of reduced cell viability towards the centre of the constructs. Overall there does appear to be more abundant green staining throughout the construct in the dynamic culture system as compared to the static. The cells were not counted because the images were from 3 dimensional constructs and the depth of the specimens was variable.

Chapter VI

Alginate Constructs in a Bioreactor

In the previous experiments the scaffold material used was agarose. The properties of agarose offer potential for the constructs to be used to assess mechanical influences on cells. It allows transmission of mechanical influences to the chondrocytes embedded within the scaffold. The use of this scaffold material was relevant for the purposes of mechanical testing but was not necessarily suitable for tissue engineering purposes. In particular there is less information available regarding its biocompatibility and biodegradability than other materials such as alginate. Although alginate has been shown to have better tissue engineering properties the problem traditionally has always been to produce disc constructs out of alginate. By the nature of its gelation, it requires the diffusion of calcium chloride through it for the cross linking to occur. Obviously to allow a satisfactory diffusion of calcium chloride and yet maintain a disc configuration it was necessary to use dialysis membrane to maintain the alginate shape while gelation occurred. This process in my experience led to inaccuracies in the diameter of the disc constructs and also resulted in the process being cumbersome and fraught with other complications ie infection etc. For these reasons I devised another simpler method of creating alginate constructs. By using alginate beads it was possible to achieve a remarkably consistent size. The beads were much smaller than the disc constructs and therefore allowed greater mass transfer of nutrients and removal of waste products. The technique employed in producing alginate beads has been described in the methods section.

6.1 Alginate Bead Constructs in a Bioreactor in short term bovine chondrocyte culture

In this experiment, the aim was to investigate the use of alginate beads for the purpose of enhancing chondrocyte proliferation in the RWV bioreactor. Chondrocytes were embedded in alginate beads and cultured for 14 days. The beads were approximately the

same size as the agarose beads. The rotating wall vessel device was compared with the static culture device. The bovine cell cultures were compared with Human cell cultures.

6.1.1 Protocol

The protocol employed was exactly the same as the protocol for the agarose bead experiment (Experiment 4).

Specimens were collected at day zero, day seven and day fourteen time points for both biochemical and histological analysis. A further set of specimens for histology were collected at day 36.

6.1.2 Results

6.1.2.1 Biochemistry

Table 6

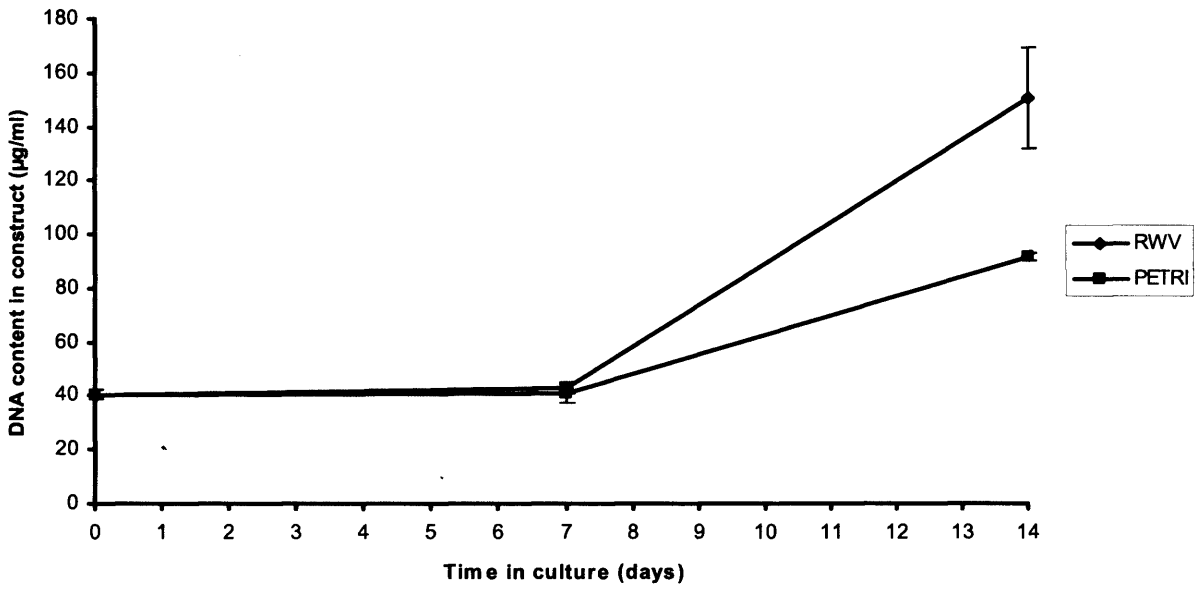
Mean DNA concentration of Alginate constructs cultured in a RWV and a Culture dish for up to 14 days

	RWV		PETRI	
Day	DNA Conc ($\mu g.ml^{-1}$)	SEM	DNA Conc ($\mu g.ml^{-1}$)	SEM
0	40.62	1.47	40.62	1.47
7	42.79	1.44	41.3	3.97
14	150.76	18.83	91.53	1.44

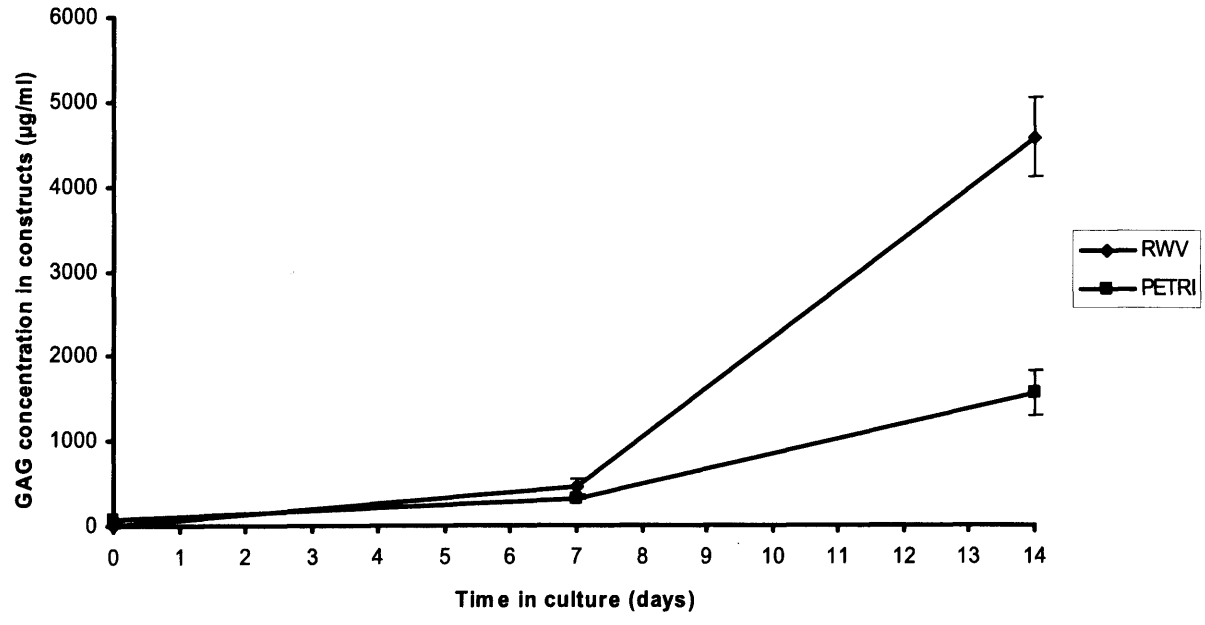
SEM = Standard Error of the Mean

Graph 11 and Table 6 show the mean DNA concentration per millilitre of alginate construct at the different time points. The initial concentration of DNA per construct was 40.62 $\mu g.ml^{-1}$ which corresponds to a cell number of 6.06 million (40.62 $x10^{-3}$ / 6.7

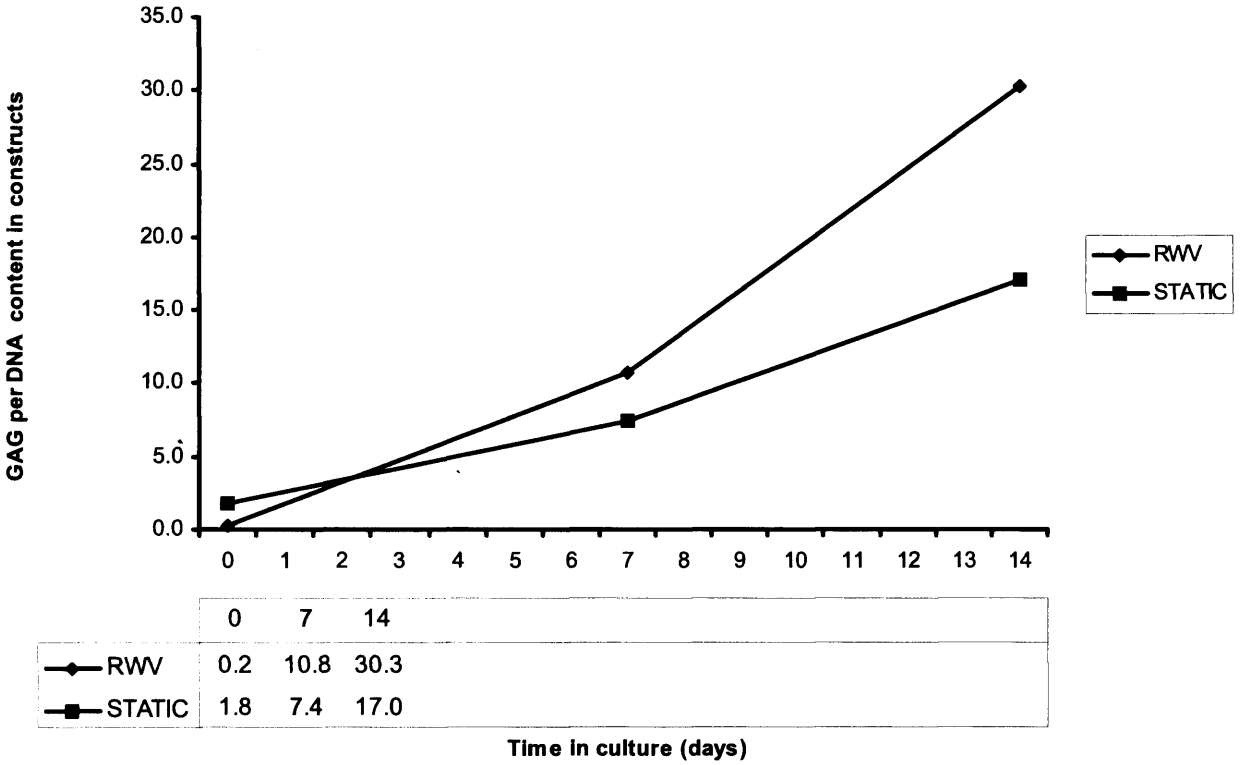
GRAPH 11. DNA content of cell seeded alginate bead constructs cultured for up to 14 days in a Dynamic Rotating Wall Vessel bioreactor and a Static Petri dish system



GRAPH 12. GAG content of cell seeded alginate bead constructs cultured forup to 14 days in a rotating wall vessel bioreactor and a Petri dish.



GRAPH 13. GAG per DNA content of cell seeded alginate bead constructs cultured for 14 days in a rotating wall vessel bioreactor and a static culture dish.



	0	7	14
RWV	0.2	10.8	30.3
STATIC	1.8	7.4	17.0

$x10^{-9}$) cells per ml of construct as each chondrocyte contains approximately 6.7 x 10^{-9} $\mu g.ml^{-1}$ DNA.

At day seven, there was a small increase to a value of $\mu g.ml^{-1}$ in the RWV and 41.3 $\mu g.ml^{-1}$ in the static device. This represents a maximum of 5% increase in both systems. The difference between the two culture systems was not significant ($p>0.05$)

At day 14, the constructs cultured in the RWV showed a DNA concentration of 150.8 $\mu g.ml^{-1}$ and the static system 91.5 $\mu g.ml^{-1}$ representing an increase in DNA concentration of 3.7 fold and 2.3 fold respectively. The difference between the two systems was also significant ($p<0.01$ Students T-Test) at this time point.

Table 7

Mean GAG concentration of Alginate constructs cultured in a RWV and a Static culture dish for up to 14 days.

	RWV		PETRI	
Day	GAG Conc ($\mu g.ml^{-1}$)	SEM	GAG Conc ($\mu g.ml^{-1}$)	SEM
0	9.7	9.4	73.7	9.4
7	461.8	93.6	305.9	0
14	4571.9	467.8	1558.1	258.7

SEM = Standard Error of the Mean

Graph 12 and Table 7 show the mean GAG concentration per millilitre of alginate construct at the specified time points of the experiment. The GAG concentration in the beads at the start of the experiment was 9.7 $\mu g.ml^{-1}$. By day 7, there was a large significant increase of GAG in both systems to a value of 461 $\mu g.ml^{-1}$ in the RWV and 306 $\mu g.ml^{-1}$ in the Static device. The GAG concentrations continue to increase and by day fourteen levels of 4572 $\mu g.ml^{-1}$ were attained in the RWV and 1558 $\mu g.ml^{-1}$ in the static device. The differences were statistically significant at both time points and between culture systems ($p<0.05$ Students T-Test). These results represent a 10 fold increase between day 7 and day 14 in the RWV and a 5 fold increase in the static culture device in GAG quantity.

Graph 13 illustrates the GAG content per DNA during the culture period. Upto day 7, the ratio increases uniformly in both culture systems and implies a steady amount of GAG production by the cells. During this period cell proliferation is similar to the agarose bead system in that it remains minimal. By day seven the ratio of GAG/DNA has increased to only 10.8 in the RWV and 7.4 in the static system as cells continue to synthesise matrix even though there is very little cell proliferation. These amounts are far less than the agarose bead cultures from the previous experiment. After day 7, cell proliferation begins to occur at an increased rate in the RWV. However, despite an increase in DNA and GAG, the overall ratio of GAG/DNA increases. This implies matrix synthesis rates are higher than cell proliferation during this phase of culture in the RWV, whereas in the static culture system, cell proliferation and GAG production maintain a steady but slower increase upto day 14. The overall DNA and GAG concentration in this system are higher than the agarose cultures.

6.1.2.2 Histology

Figure 6.1.1 shows an alginate bead construct at day seven in the static culture system. The chondrocytes are well distributed within the bead and on close inspection there is evidence of early matrix production. There are no cell clusters at this stage. *Figure 6.1.2 and 6.1.3* is a Safranin-O staining at the same time point. These specimens demonstrate evidence of only minimal matrix production. The chondrocytes do however appear rounded and healthy.

Figure 6.1.4 to 6.1.6 show the response of alginate bead cell cultures to the RWV. The cells all appear much more healthy than in the case of the static cultures. There are occasional cell clumps with abundant extracellular matrix. The cell nuclei are larger and appear more active. The Safranin-O stains intensely around the cells demonstrating a significant deposition of peri-cellular matrix. High magnification demonstrates the clarity of the staining of the matrix.

Figure 6.1.7 to 6.1.14 illustrates representative histograms prepared from the periphearl and central regions of bovine chondrocyte-seeded alginate constructs maintained in

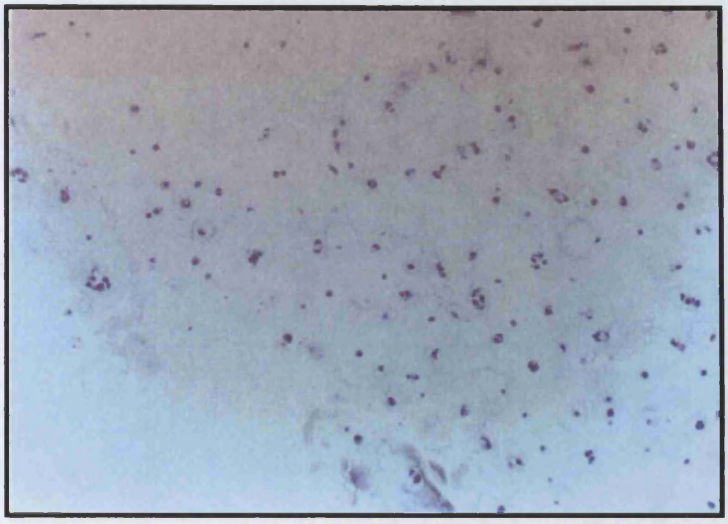


Figure 6.1.1
H&E Stain. Magn X10.
Alginate bead construct cultured in the Static system for 7 days.

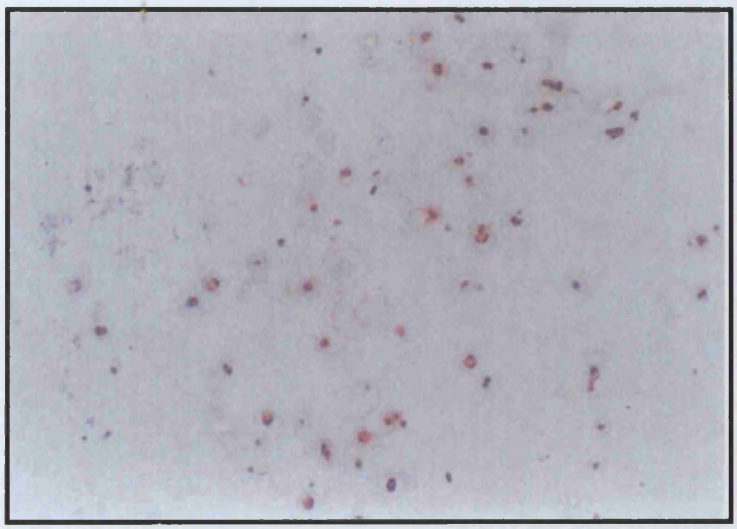


Figure 6.1.2
Saf-O Stain. Magn X10.
Edge of Alginate bead construct cultured in the Static system for 7 days.

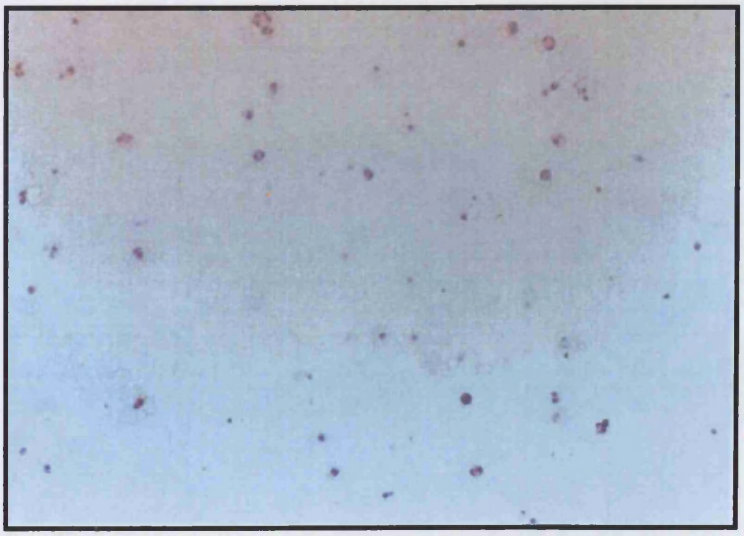


Figure 6.1.3
Saf-O Stain. Magn X10.
Centre of Alginate bead construct cultured in the Static system for 7 days.

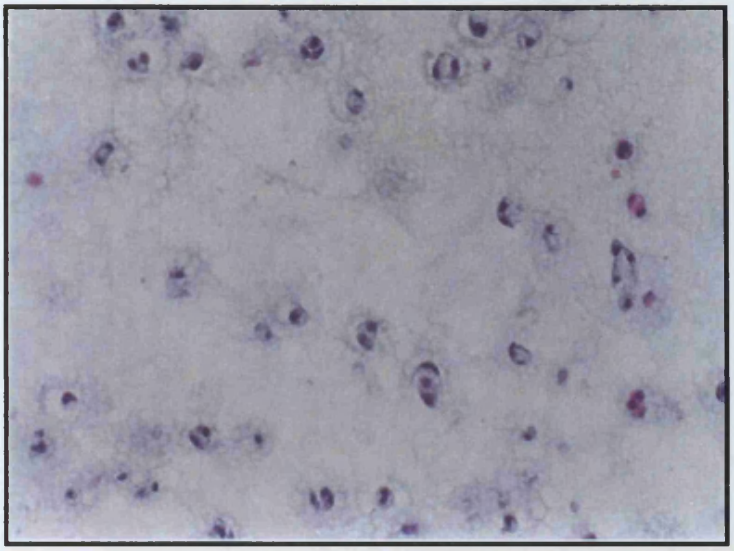


Figure 6.1.4
H&E Stain. Magn X20.
Alginate bead
construct cultured in the
RWV system for 7 days.

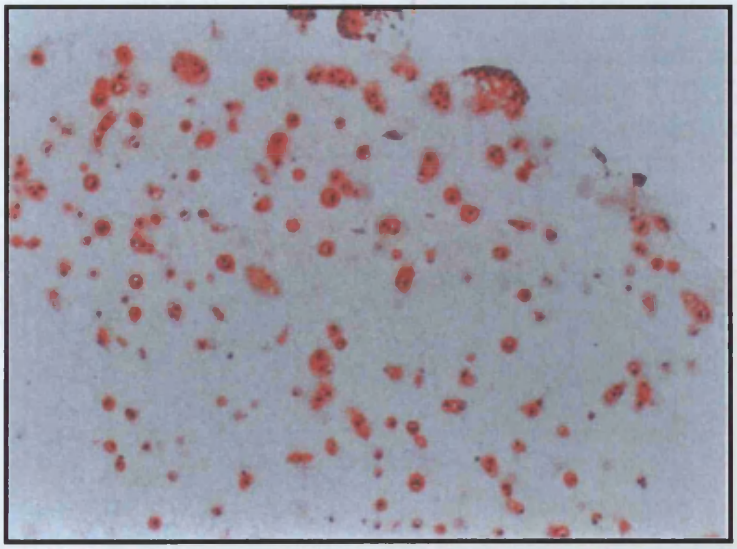


Figure 6.1.5
Saf-O Stain. Magn X10.
Alginate bead
construct cultured in the
RWV system for 7 days.

Figure 6.1.6
Saf-O Stain. Magn X100.
Alginate bead. Clump
of cells at 7 days in the
RWV system.

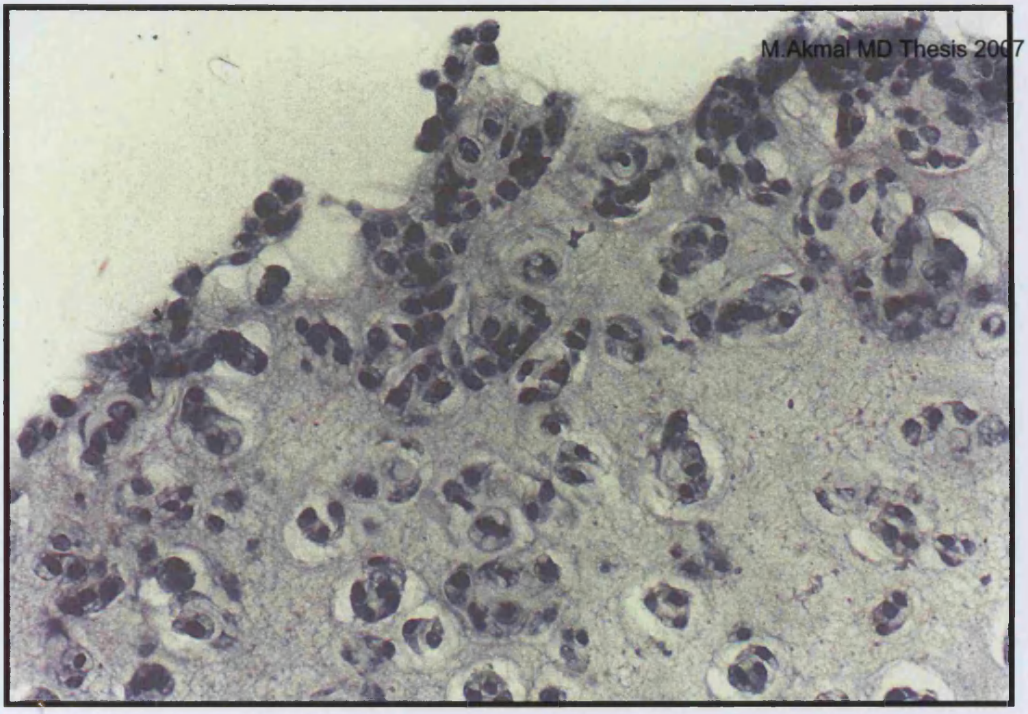


Figure 6.1.7 H&E Stain. Magn X20. Edge. Alginate bead
Day 14 culture in Static system

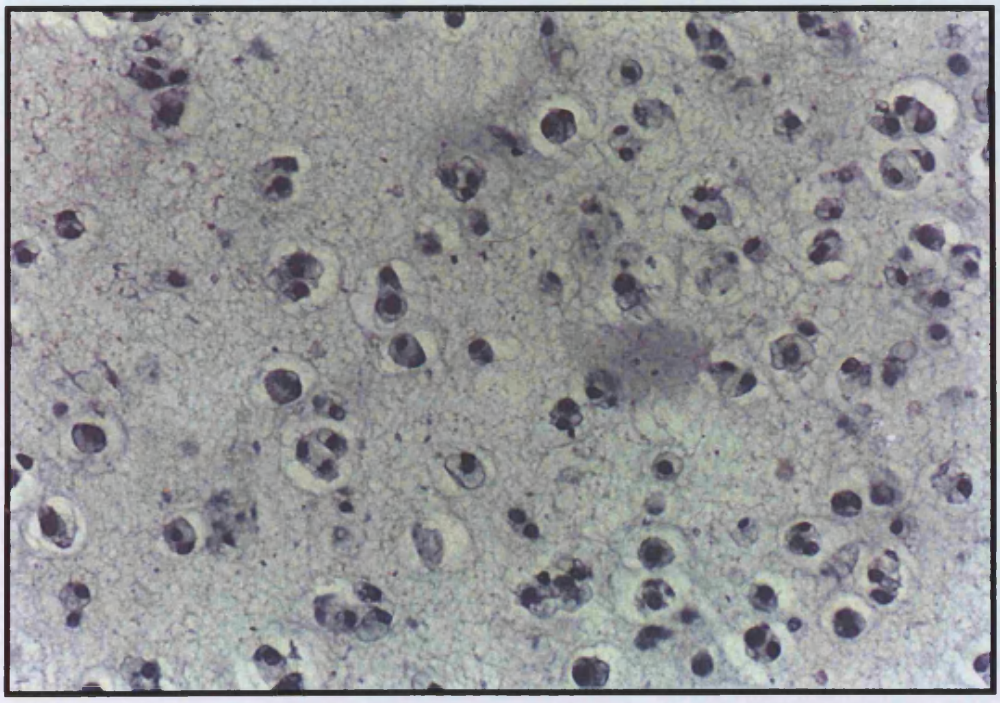


Figure 6.1.8 H&E Stain. Magn X20. Centre. Alginate bead
Day 14 culture in RWV system

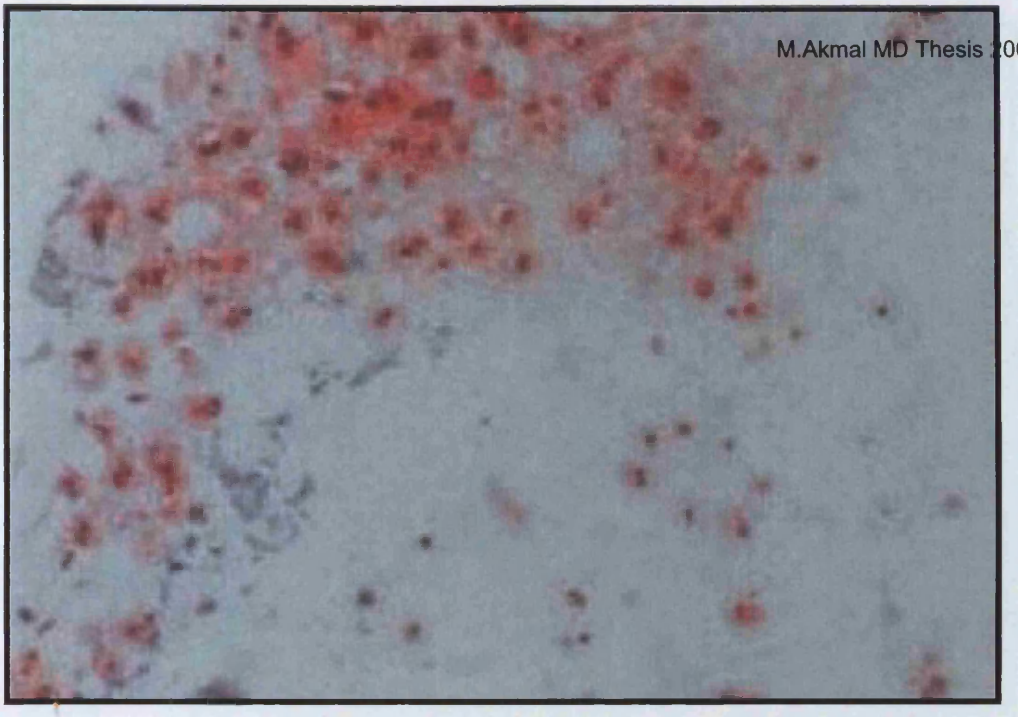


Figure 6.1.9 Safranin-O Stain. Magn X20. Edge. Alginate bead Day 14 culture in Static system

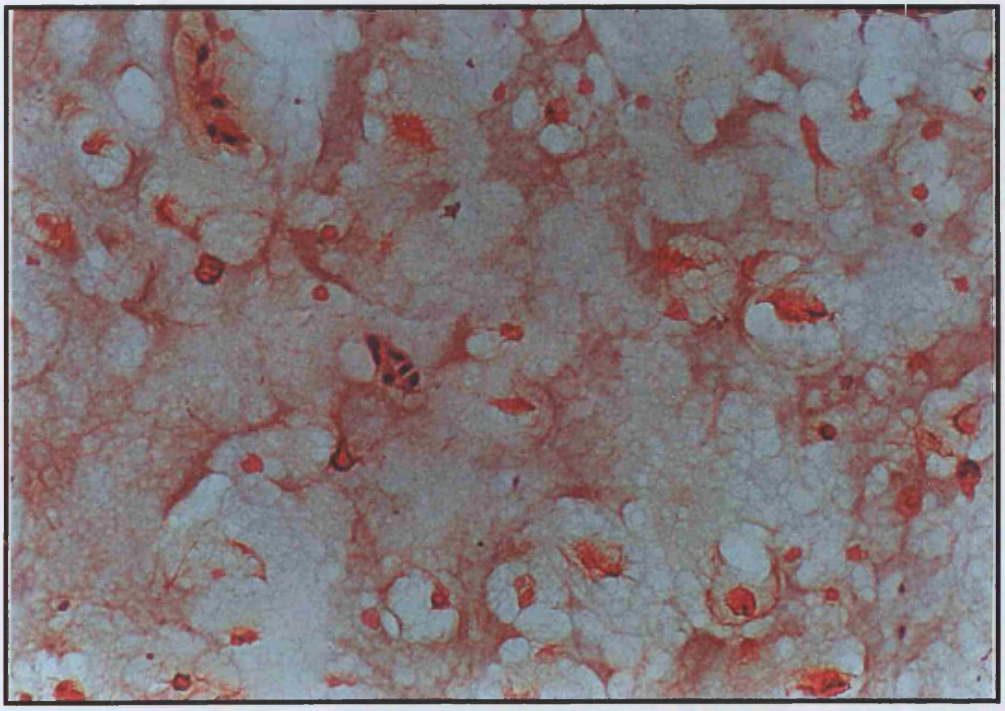


Figure 6.1.10 Safranin-O Stain. Magn X20. Centre. Alginate bead Day 14 culture in Static system

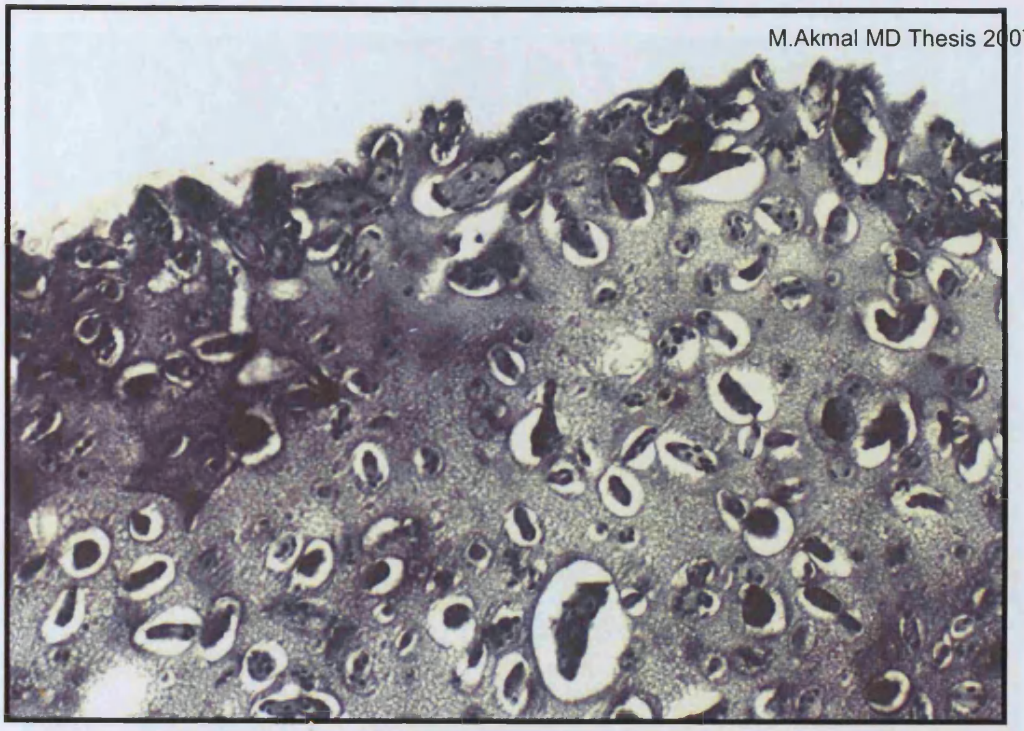


Figure 6.11 H&E Stain. Magn X10. Edge. Alginate bead
Day 14 culture in RWV system

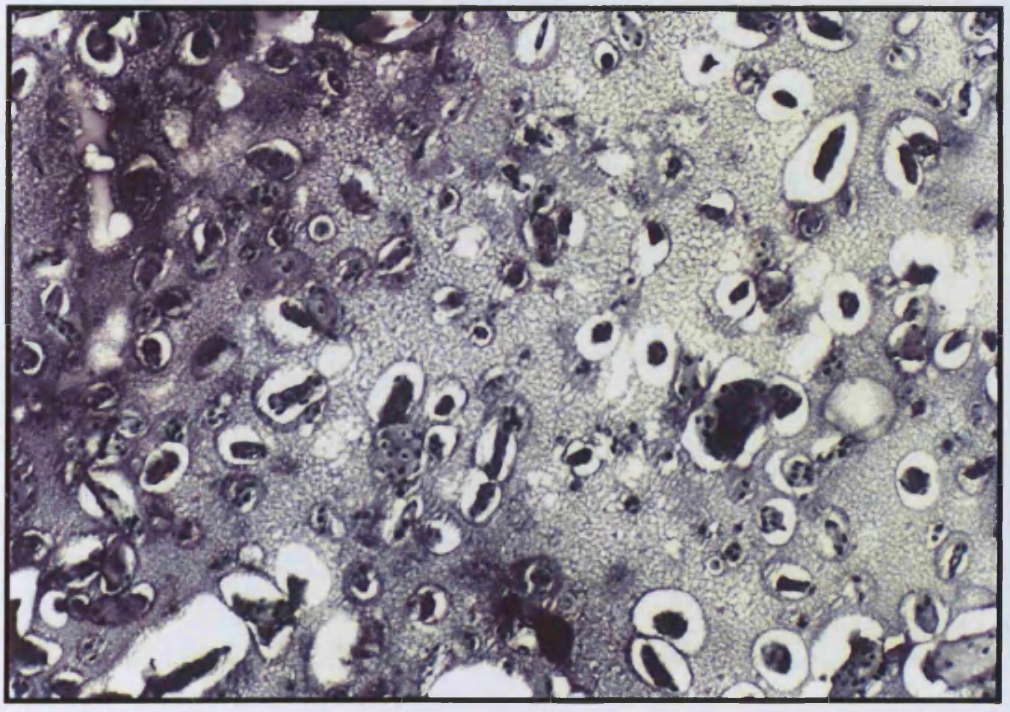


Figure 6.12 H&E Stain. Magn X10. Centre. Alginate bead
Day 14 culture in RWV system

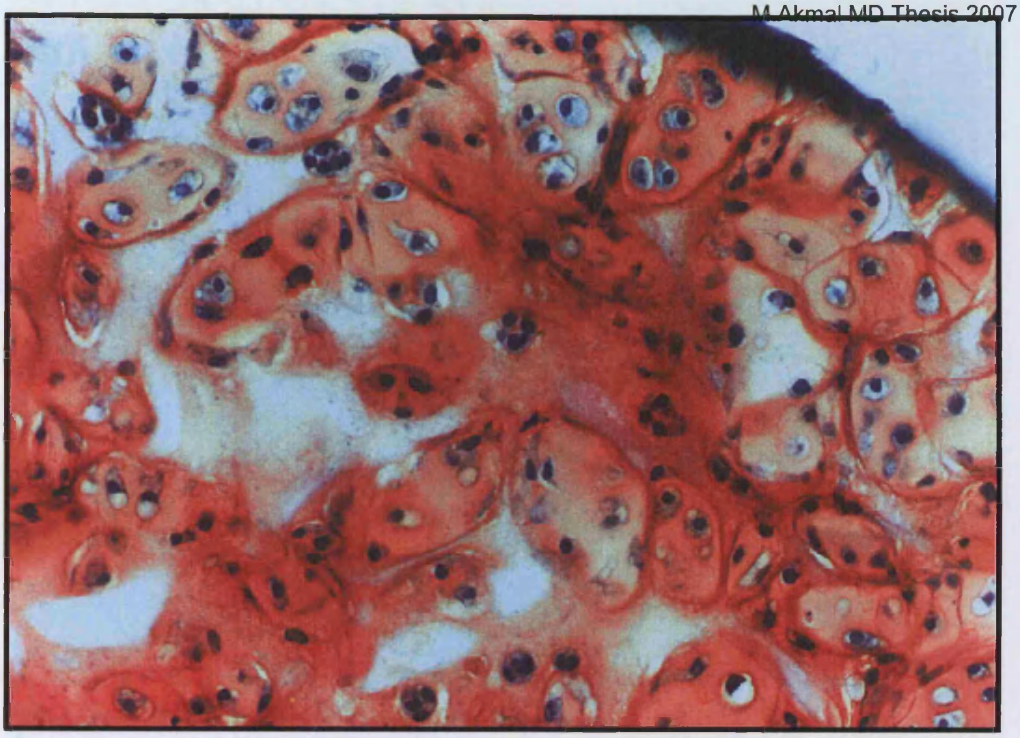


Figure 6.1.13 H&E Stain. Magn X20. Edge. Alginate bead
Day 14 culture in RWV system

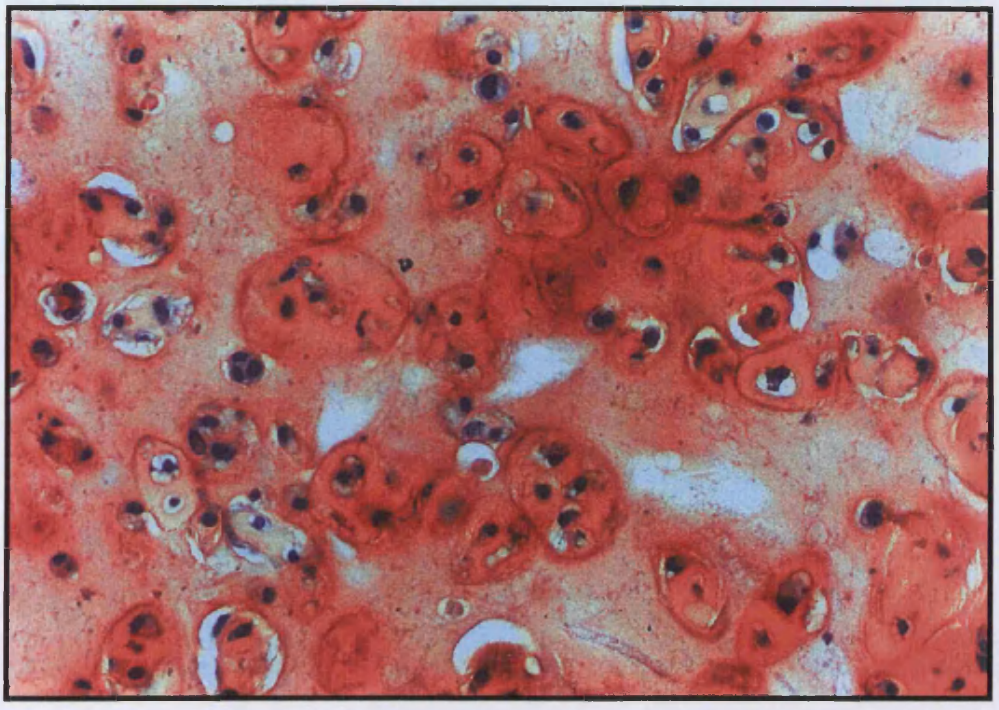


Figure 6.1.14 H&E Stain. Magn X20. Centre. Alginate bead
Day 14 culture in RWV system

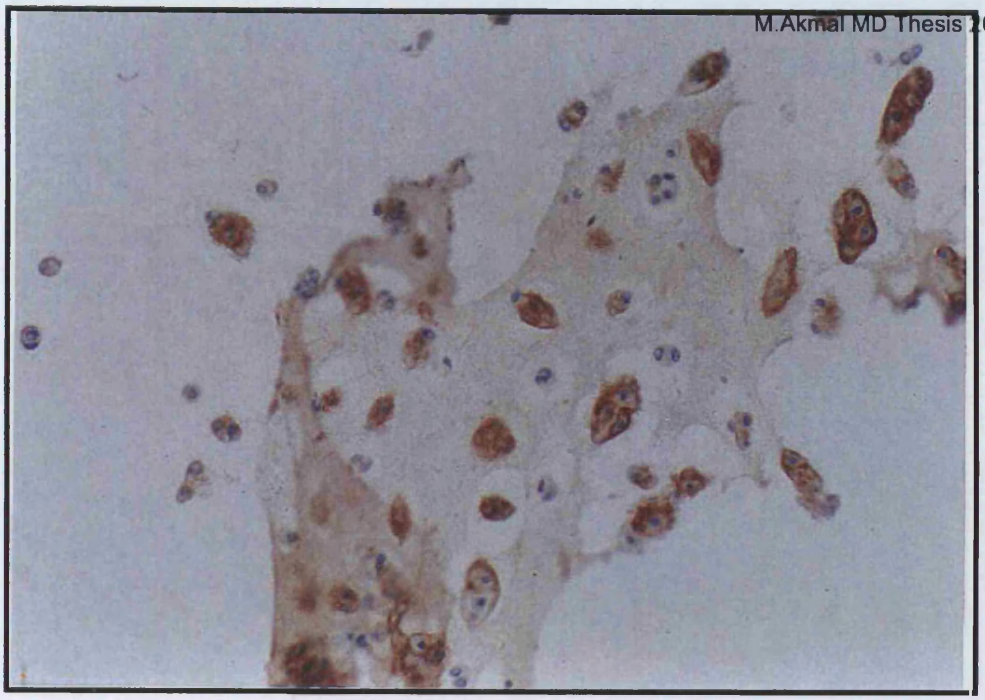


Figure 6.1.15 Collagen Type II Immunolabelling. Magn X10.
Alginate bead construct cultured in the Static system for 14 days.

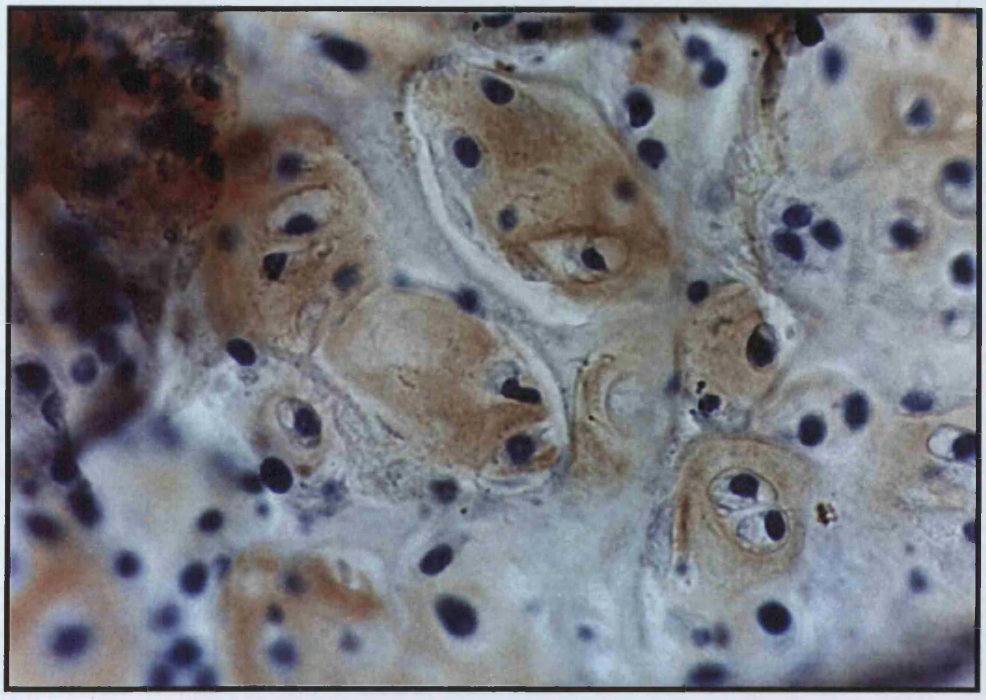


Figure 6.1.16 Collagen type II immunolabelling. Magn X20.
Alginate bead construct cultured in the RWV system for 14 days.

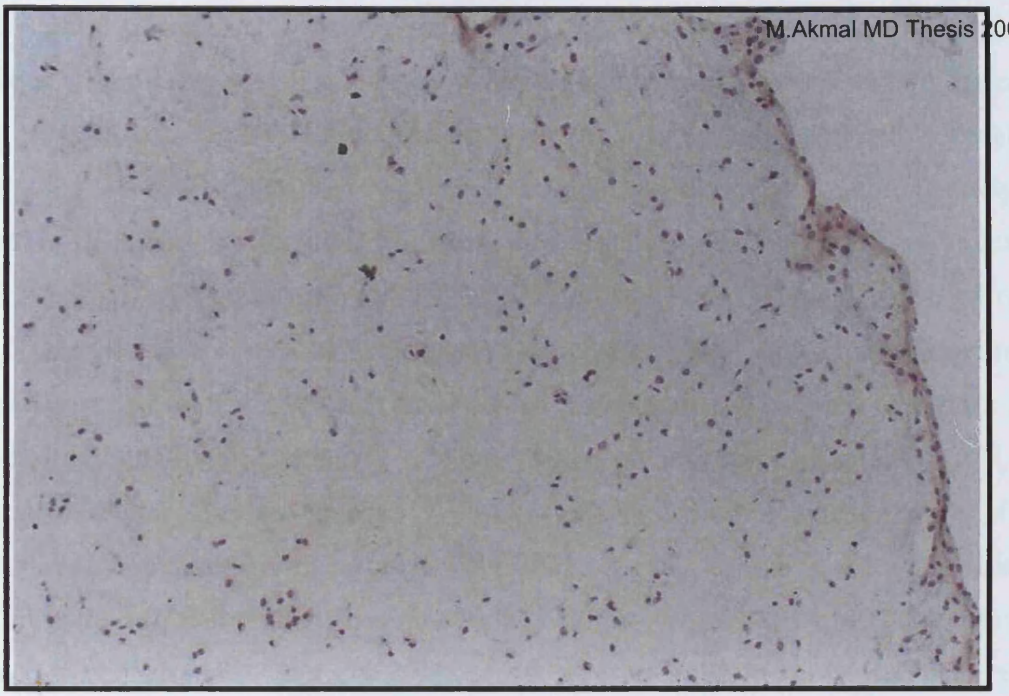


Figure 6.1.17 Collagen Type I Immunolabelling. Magn X10.
Alginate bead construct cultured in the Static system for 14 days.

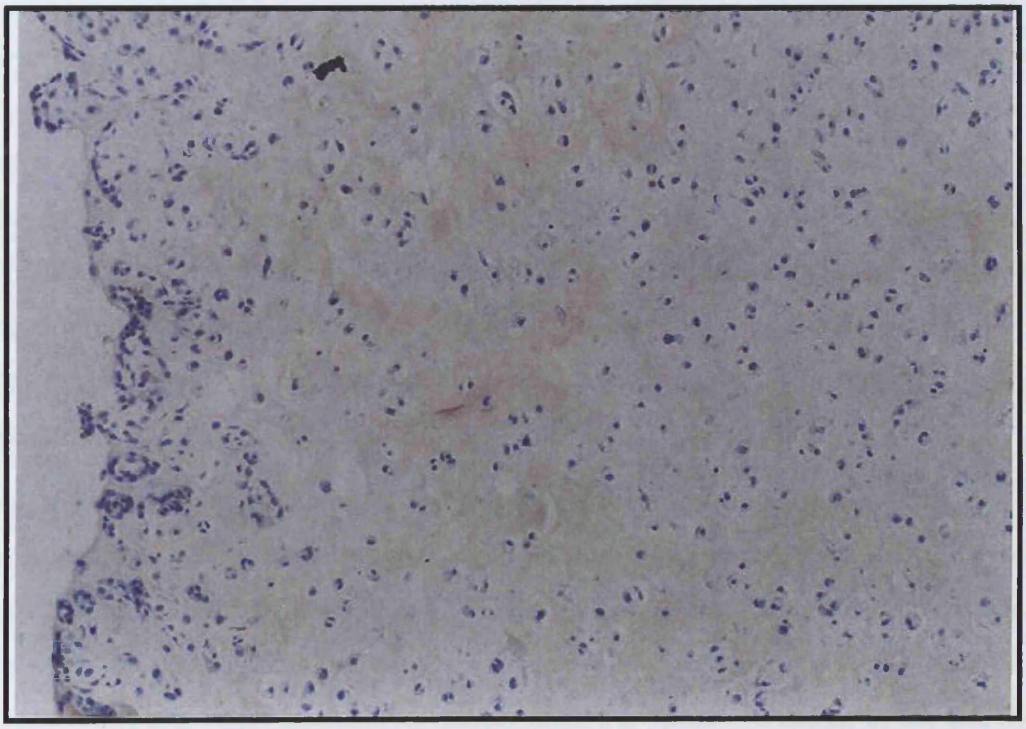


Figure 6.1.18 Collagen type VI immunolabelling. Magn X10.
Alginate bead construct cultured in the RWV system for 14 days.

static culture (*figures 6.1.7 to 6.1.10*) or within the RWV (*figures 6.1.11 to 6.1.14*) for 14 days. In the peripheral region of constructs maintained in static culture the cells were well dispersed, existed in groups of up to 4 cells and exhibited a rounded morphology. A pericellular region of GAG-rich extracellular matrix, approximately 5-10 μm in thickness, was observed to surround the majority of cell groups, as indicated by Safranin-O staining. At the extreme periphery, however, a layer of flattened cells was present which formed a capsule around the construct. By contrast, at the centre of the construct no groups of cells were observed and Safranin-O staining was weak and present only in the immediate pericellular space. When cultured in the RWV, an increase in the intensity and extent of Safranin-O staining was noted in all regions of the construct as compared to static conditions. Three regions of matrix were identifiable and, in some locations, neighbouring clumps of inter-territorial matrix had combined. Moreover cell clusters often consisted of 10 or more cells in the peripheral region (*Figure 6.1.13*) and up to 4 cells in the central region. The cells at the periphery were larger and exhibited enhanced Safranin-O staining compared to cells in the central region. No capsule of flattened cells was present surrounding constructs cultured in the RWV bioreactor.

Figures 6.1.16 to 6.1.18 illustrate immunolocalisation within constructs of collagen type II, Type I and Type VI respectively cultured in RWV Bioreactor conditions. Bovine chondrocytes cultured in static conditions exhibited limited type II collagen staining (*figure 6.1.15*), primarily associated with rounded cells at the periphery of the construct. The cells at the surface of the construct with a flattened morphology did not express collagen type II. In the RWV culture conditions, the staining for collagen type II was of greater intensity and distributed more extensively throughout the construct, when compared to static conditions.

Type I collagen was expressed only by cells with a flattened morphology, which formed a capsule surrounding chondrocyte-seeded constructs cultured in static conditions (*Figure 6.1.16*). No type I collagen expression was observed for constructs maintained in the RWV bioreactor (data not shown). Type X collagen staining was not detected in any constructs, although the positive control exhibited intense staining in the calcified cartilage region (data not shown). Type VI collagen was detected in the RWV specimens at day 14 (*figure 6.1.18*).

6.2 Alginate Bead Constructs in a Bioreactor in Long term bovine chondrocyte culture

6.2.1 Protocol for Long Term Bovine Chondrocyte Cultures

The protocol employed was exactly the same as the protocol for the agarose bead experiment (Section 5.2.1). However, the medium was changed every 3 days and the cultures were maintained for 29 days.

Specimens were collected at day zero, day three, day seven, day ten, day fourteen, day twenty and day twenty nine time points for both biochemical and histological analysis.

6.2.2 Results

6.2.2.1 Biochemistry

Table 8

Mean DNA concentration of Alginate constructs cultured in a RWV and a Culture dish for up to 29 days

	RWV		PETRI	
Day	DNA Conc ($\mu g.ml^{-1}$)	SEM	DNA Conc ($\mu g.ml^{-1}$)	SEM
0	44.59	3.26	55.69	2.48
3	60.16	1.44	59.54	3.96
7	92.31	2.82	75.94	2.00
10	94.20	2.11	75.80	5.92
14	201.03	7.80	144.79	8.57
17	305.34	1.95	136.70	3.30
20	278.75	17.42	127.56	16.34
29	388.32	3.59	197.9	10.86

SEM = Standard Error of the Mean

Graph 14 and Table 8 show the mean DNA concentration per millilitre of alginate construct at the different time points. The initial concentration of DNA per construct was 44.59 $\mu g.ml^{-1}$ which corresponds to a cell number of 6.65 million (44.59 $x10^{-3}$ / 6.7 $x10^{-9}$) cells per ml of construct as each chondrocyte contains approximately 6.7 x 10^{-9} $\mu g.ml^{-1}$ DNA.

At day three and day seven, there was a small increase to a value of 92.3 $\mu g.ml^{-1}$ in the RWV and 75.94 $\mu g.ml^{-1}$ in the static device. This represents a 2 fold increase in the RWV and 50% increase in the static system. The difference between the two culture systems was significant at all time points after 7 days ($p>0.01$)

By day 14, the differences between the RWV and the static device were very apparent. The RWV mainitained a much higher rate of cell proliferation throughout the remainder of the culture period to a peak of 388 $\mu g.ml^{-1}$ by day 29. This represents a greater than 8 fold increase in cell numbers in the RWV. The DNA proliferation was not as marked in the static device, with only a modest increase to 197.9 $\mu g.ml^{-1}$ by day 29. This represents a 4 fold increase.

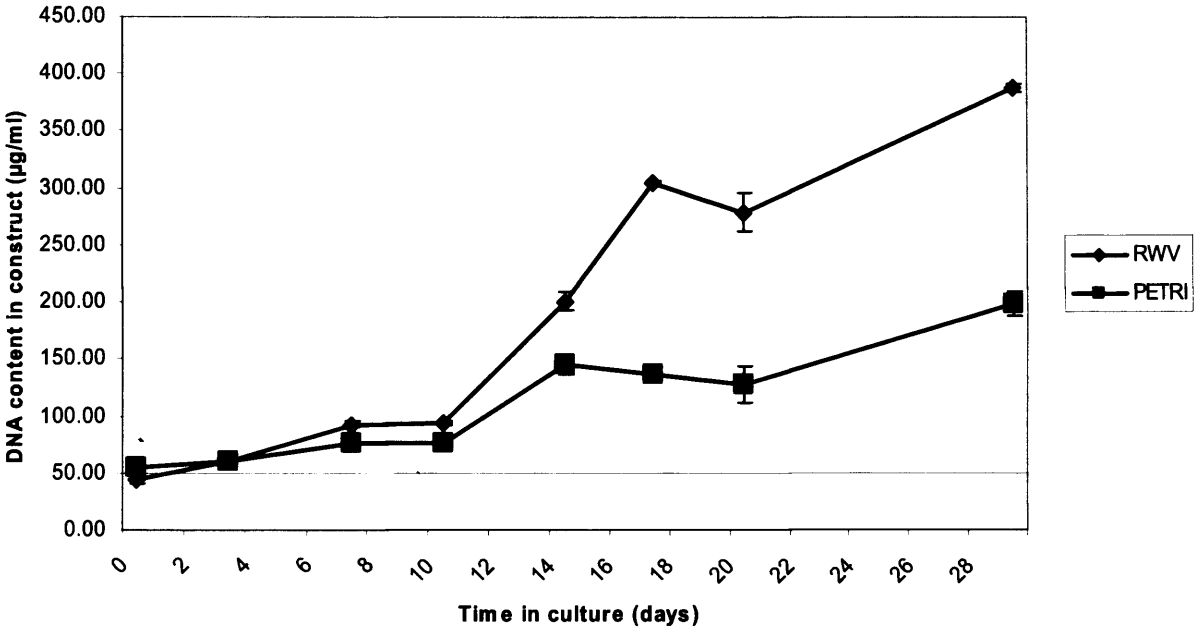
Table 9

Mean GAG concentration of Alginate constructs cultured in a RWV and a Static culture dish for up to 29 days.

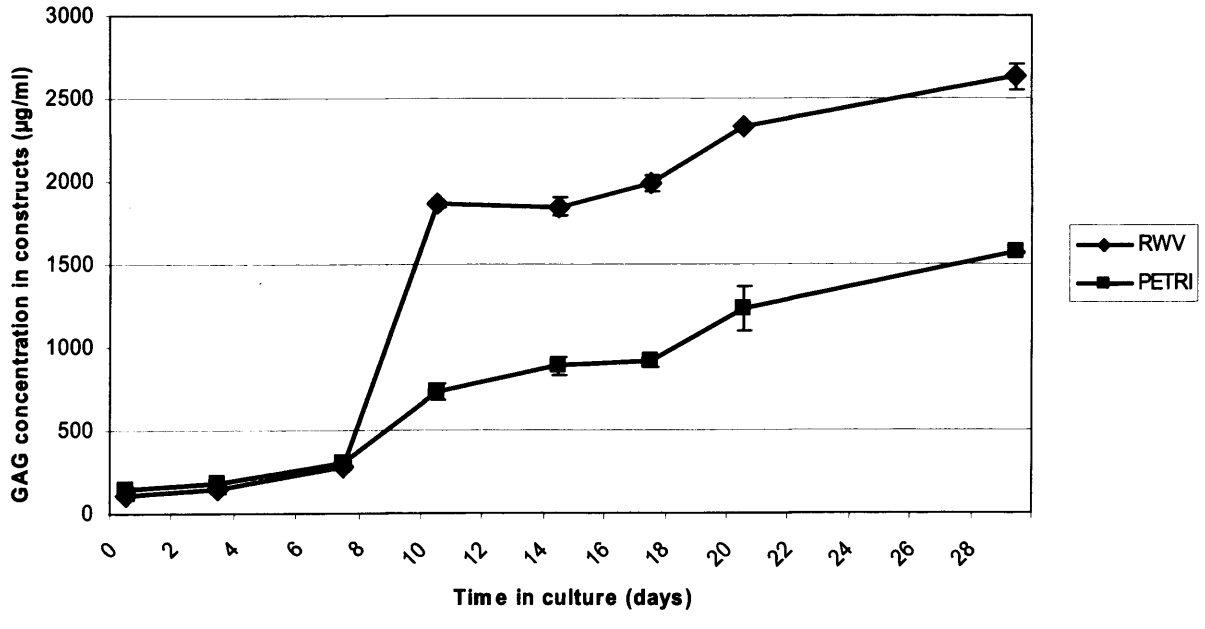
	RWV		PETRI	
Day	GAG Conc ($\mu g.ml^{-1}$)	SEM	GAG Conc ($\mu g.ml^{-1}$)	SEM
0	104.22	13.6	150.58	13.60
3	141.06	20.17	184.74	10.00
7	285.46	15.55	306.78	13.61
10	1861.10	20.09	734.34	50.73
14	1846.05	54.39	887.83	54.82
17	1984.18	49.02	913.15	33.83
20	2327.03	11.08	1229.57	137.66
29	2629.44	82.72	1571.53	4.79

SEM = Standard Error of the Mean

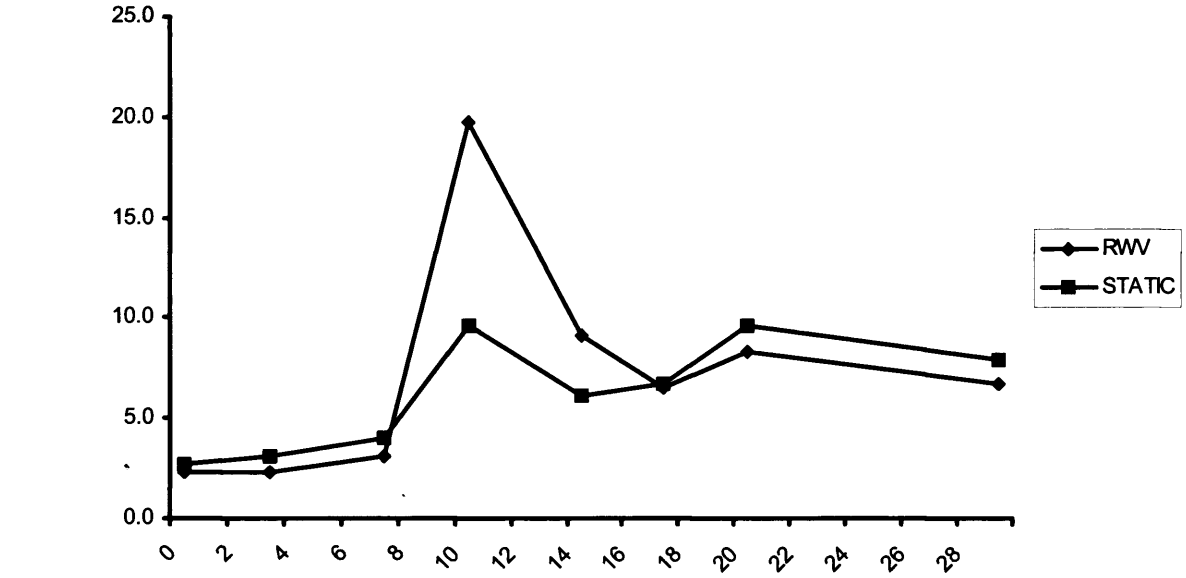
GRAPH 14. DNA content of Bovine chondrocyte seeded alginate beads cultured for up to 29 days in a Dynamic Rotating Wall Vessel bioreactor and a Static Petri dish system (10 beads)



GRAPH 15. GAG content of Bovine chondrocyte seeded alginate bead constructs cultured for up to 29 days in a Rotating Wall Vessel Bioreactor and a Static culture dish (10 beads).



GRAPH 16. GAG per DNA content of cell seeded alginate bead constructs cultured for 29 days in a rotating wall vessel bioreactor and a static culture dish.



	0	3	7	10	14	17	20	29
RWV	2.3	2.3	3.1	19.8	9.2	6.5	8.3	6.8
STATIC	2.7	3.1	4.0	9.7	6.13	6.68	9.64	7.94

Time in culture (days)

Graph 15 and Table 9 show the mean GAG concentration per millilitre of alginate construct at the specified time points of the experiment. By day 7, there was a large significant increase of GAG in both systems to a value of 285 $\mu g.ml^{-1}$ in the RWV and 306 $\mu g.ml^{-1}$ in the Static device. The GAG concentrations continue to increase and by day ten levels of 1861 $\mu g.ml^{-1}$ were attained in the RWV and 734 $\mu g.ml^{-1}$ in the static device. By day 29, the Gag levels reached 2629 $\mu g.ml^{-1}$ in the RWV and 1571 $\mu g.ml^{-1}$ in the RWV. The differences were statistically significant at both time points and between culture systems ($p<0.05$ Students T-Test). These results represent a 9 fold increase between day 7 and day 29 in the RWV and a 5 fold increase in the static culture device in GAG quantity.

Graph 16 illustrates the GAG content per DNA during the 29 day culture period. Upto day 8, the ratio increases uniformly in both culture systems and implies a small steady amount of GAG production by the cells. By day 8 the ratio of GAG/DNA has increased to only 3 in the RWV and 4 in the static system. Between day 8 and day 12 there is a rapid increase in cell proliferation and GAG production in the RWV as compared to the static culture. The GAG synthesis is proportionally higher in the RWV during this period and is represented in this graph. Between day 12 and day 18, the ratio of GAG/DNA falls in both systems implying a period of cell proliferation rather than matrix synthesis. After day 18, there is again a small increase in GAG/DNA peaking at day 20, but the general trend remains consistent with a similar GAG/DNA ratio in both systems upto the end of the experiment. Cell proliferation and GAG production continues to increase in both systems but at higher rates in the RWV during this period.

6.2.2.2 Histology

Histological examination demonstrates appearances in cellular morphology and extracellular matrix production that are consistent with the biochemical findings. *Figures 6.2.1-6.2.15* illustrate histograms prepared from the central and peripheral region of bovine chondrocyte-seeded alginate constructs maintained in static culture or within the RWV for up to 36 days.

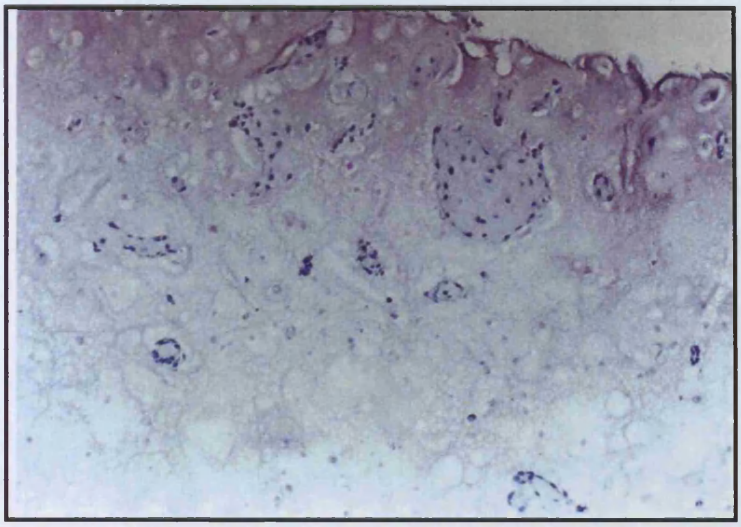


Figure 6.2.1
H&E Stain. Magn X10.
Alginate bead
construct cultured in the
RWV system for 21 days.

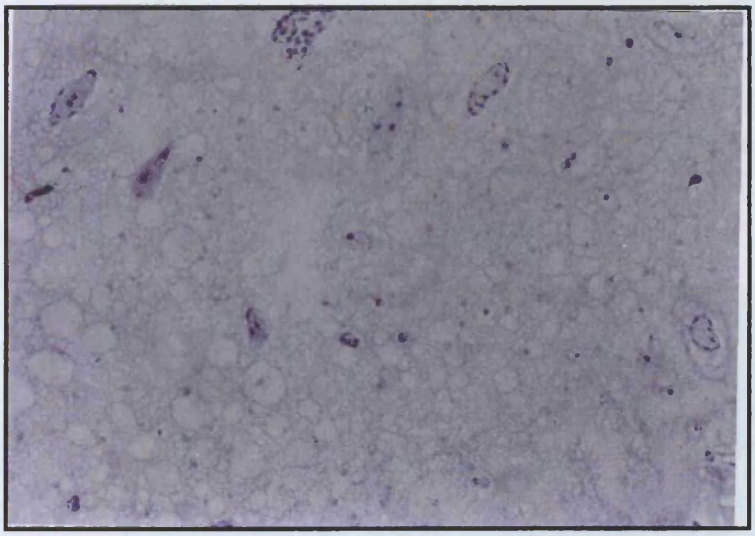


Figure 6.2.2
H&E Stain. Magn X10.
Alginate bead
construct cultured in the
Static system for 21 days.

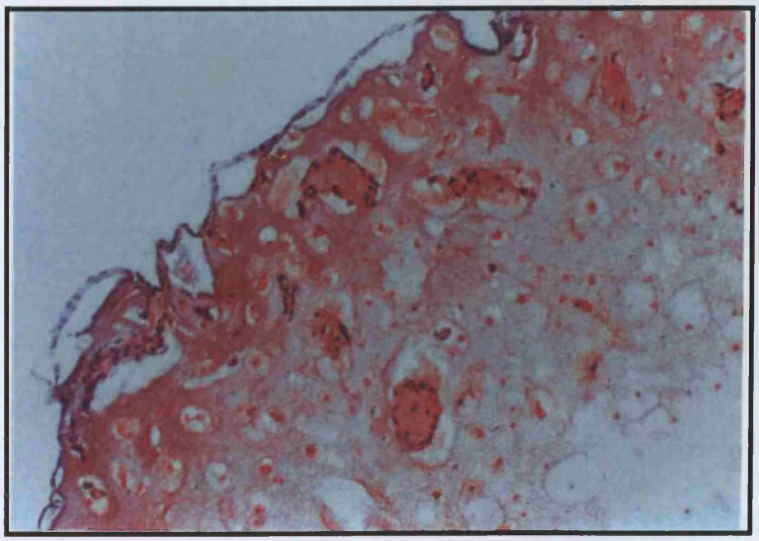


Figure 6.2.3
Saf-O Stain. Magn X10.
Alginate bead construct
cultured for 21 days in the
Static system.

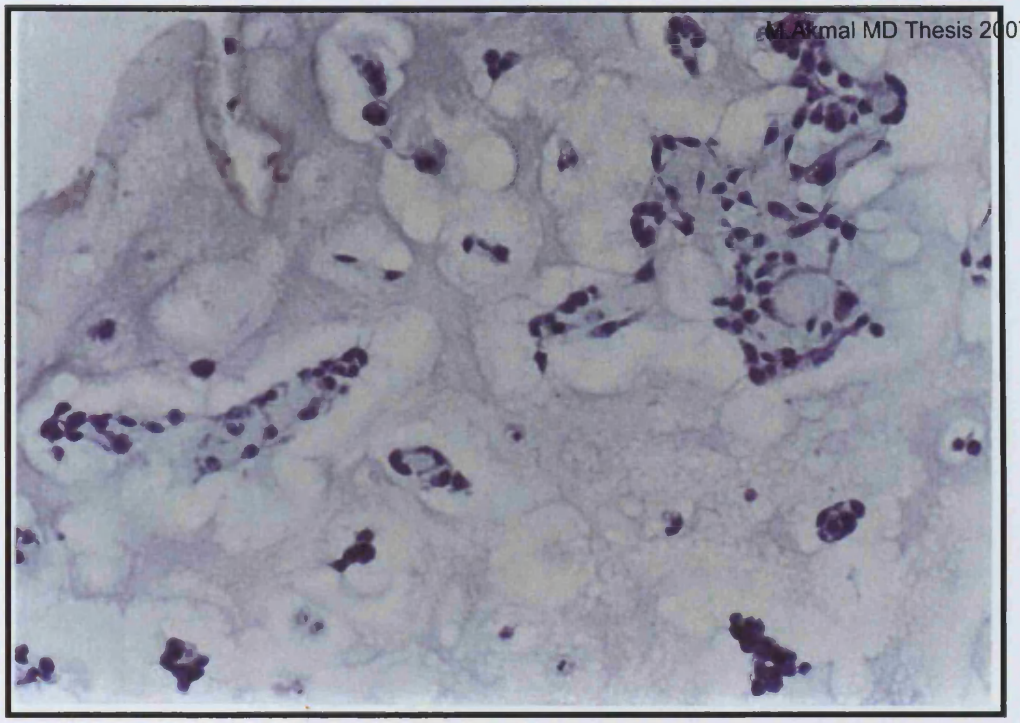


Figure 6.2.4 H&E Stain. Magn X20. Edge. Alginate bead
Day 21 culture in RWV system

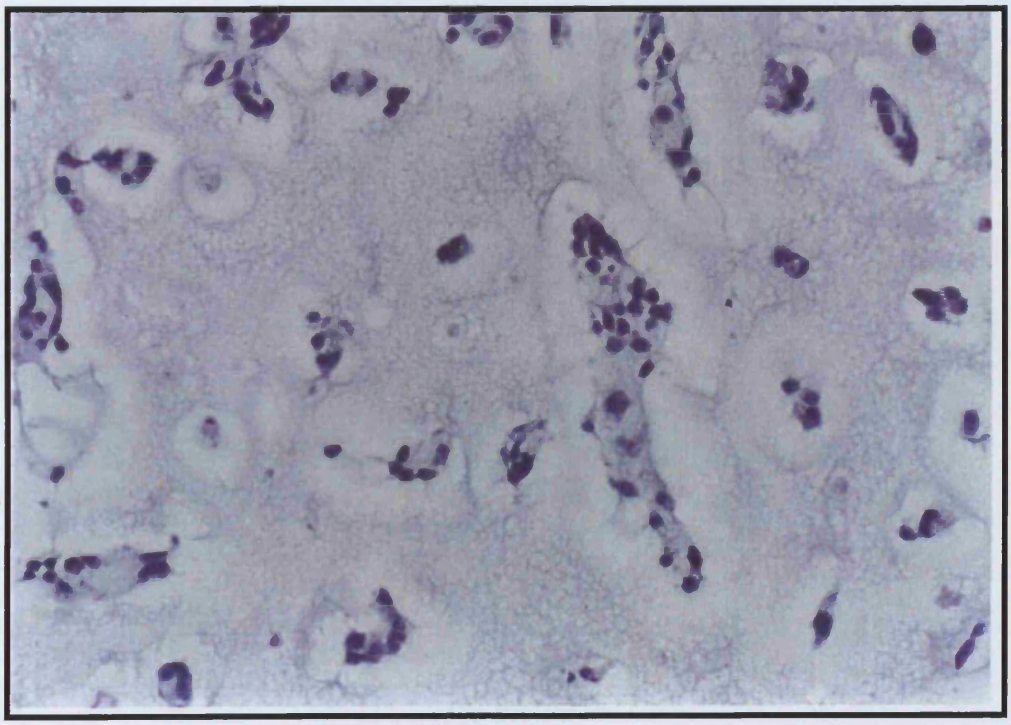


Figure 6.2.5 H&E Stain. Magn X20. Centre. Alginate bead
Day 21 culture in RWV system

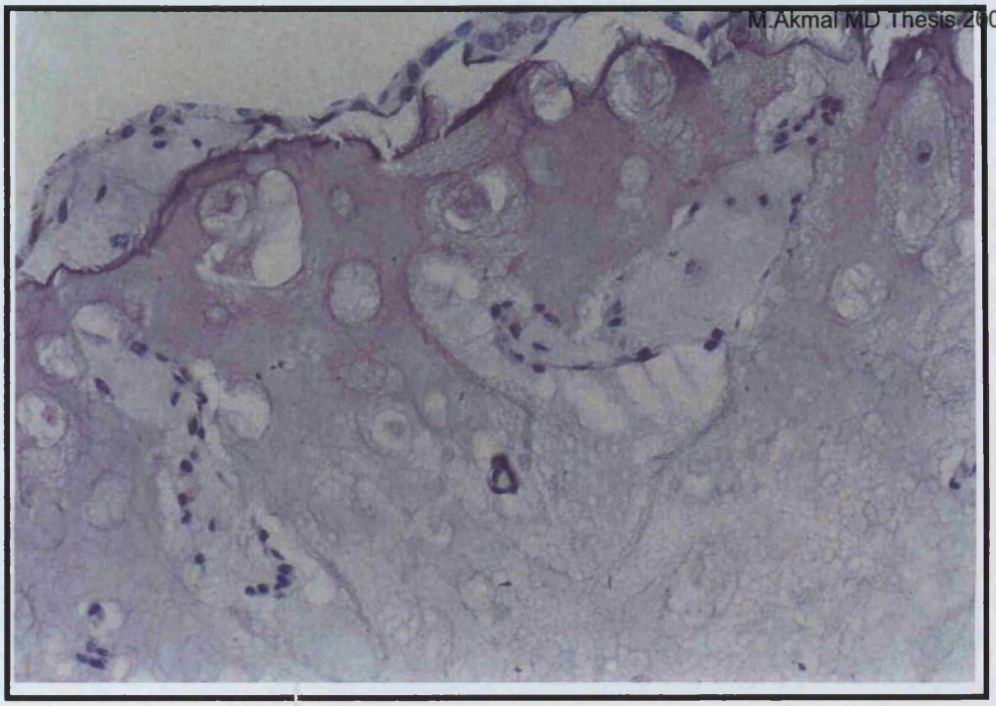


Figure 6.2.6 H&E Stain. Magn X20. Edge. Alginate bead
Day 29 culture in Static system

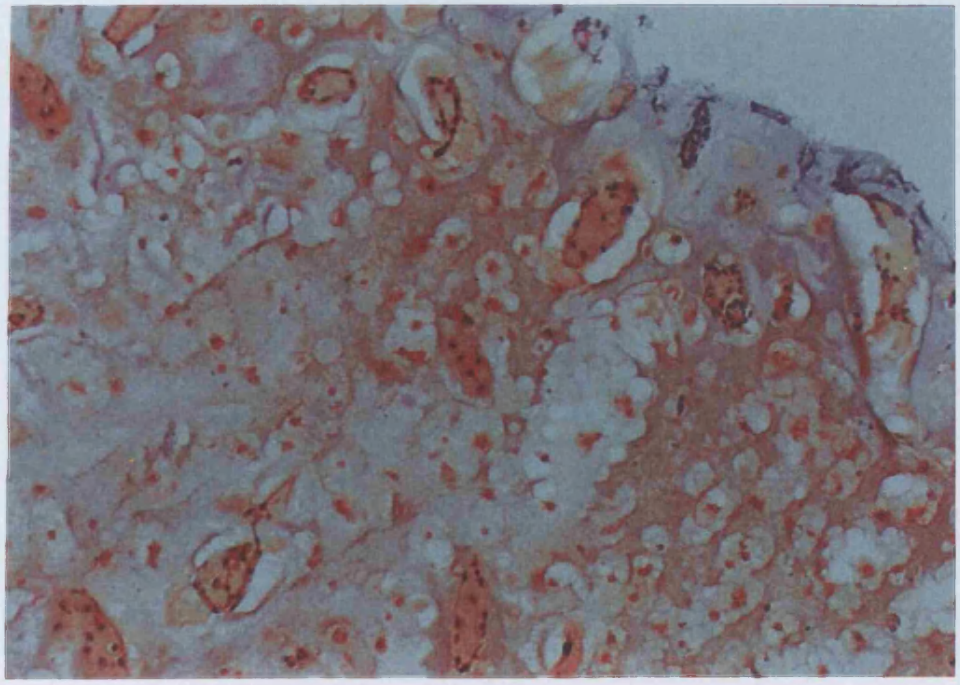


Figure 6.2.7 H&E Stain. Magn X10. Centre. Alginate bead
Day 29 culture in Static system

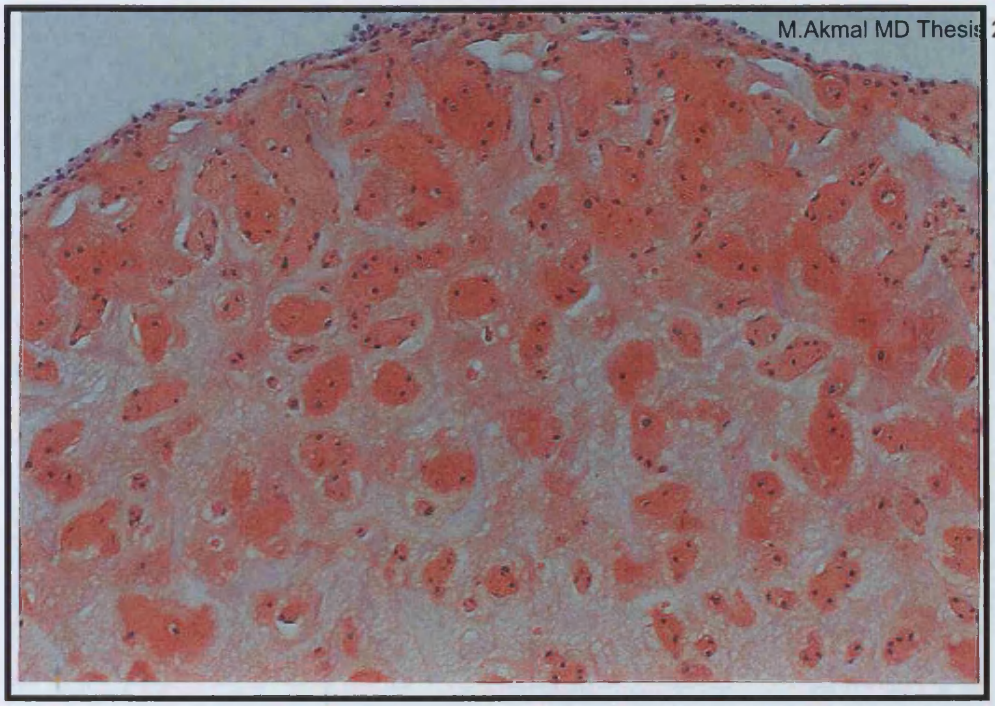


Figure 6.2.8 Safranin-O Stain. Magn X10.
Edge of Alginate bead
Day 29 culture in the RWV system.

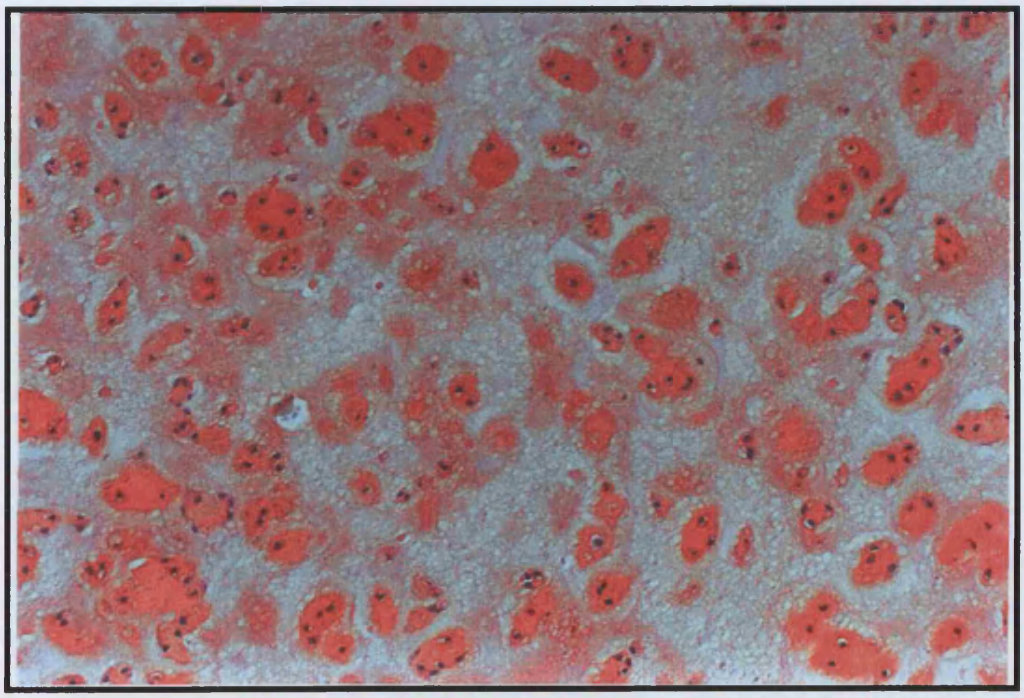


Figure 6.2.9 Safranin-O Stain. Magn X10.
Centre of Alginate bead
Day 29 culture in the RWV system.

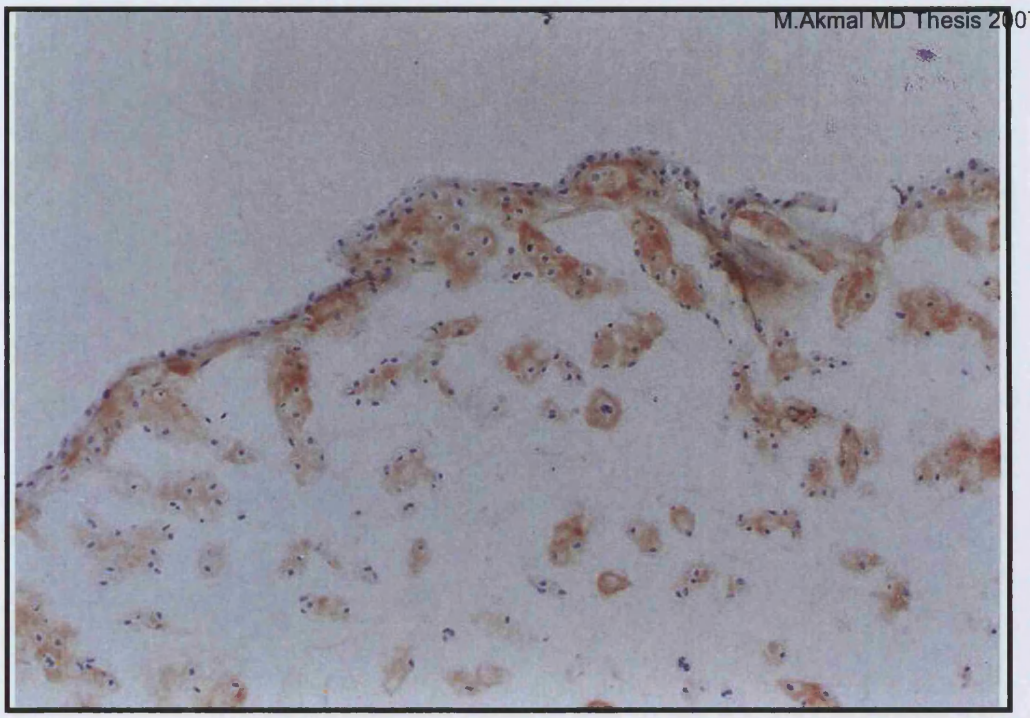


Figure 6.2.10 Collagen type II Immunolabelling. Magn X10.
Alginate bead. Day 29 culture in Static system

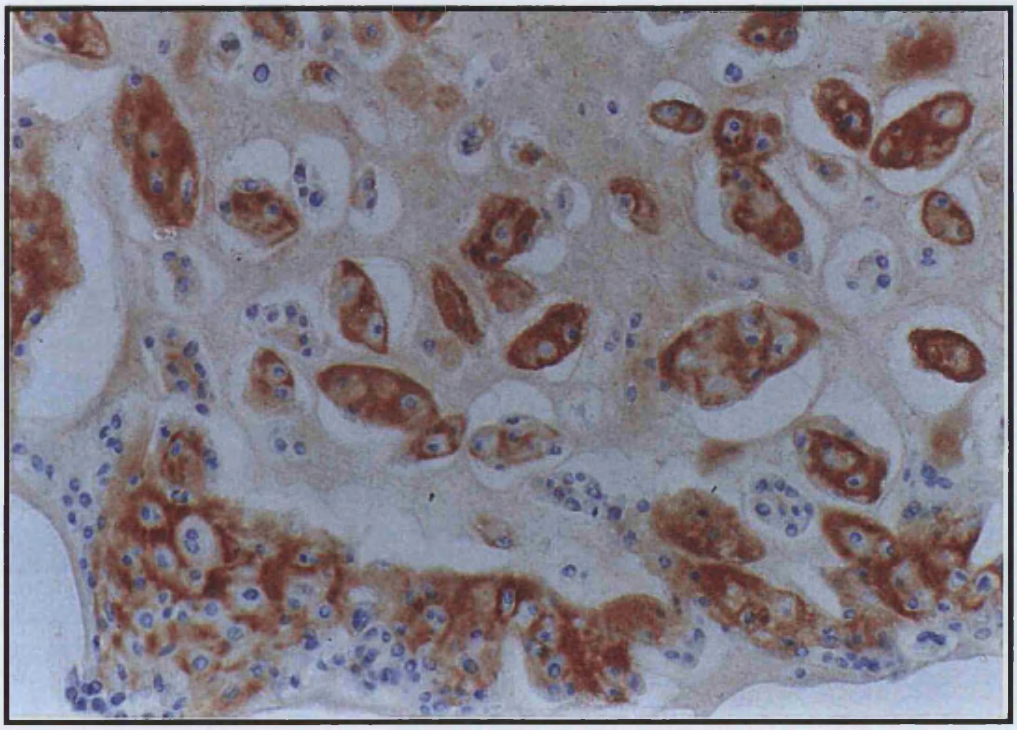


Figure 6.2.11 Collagen Type II immunolabelling. Magn X20. Centre.
Alginate bead. Day 29 culture in RWV system

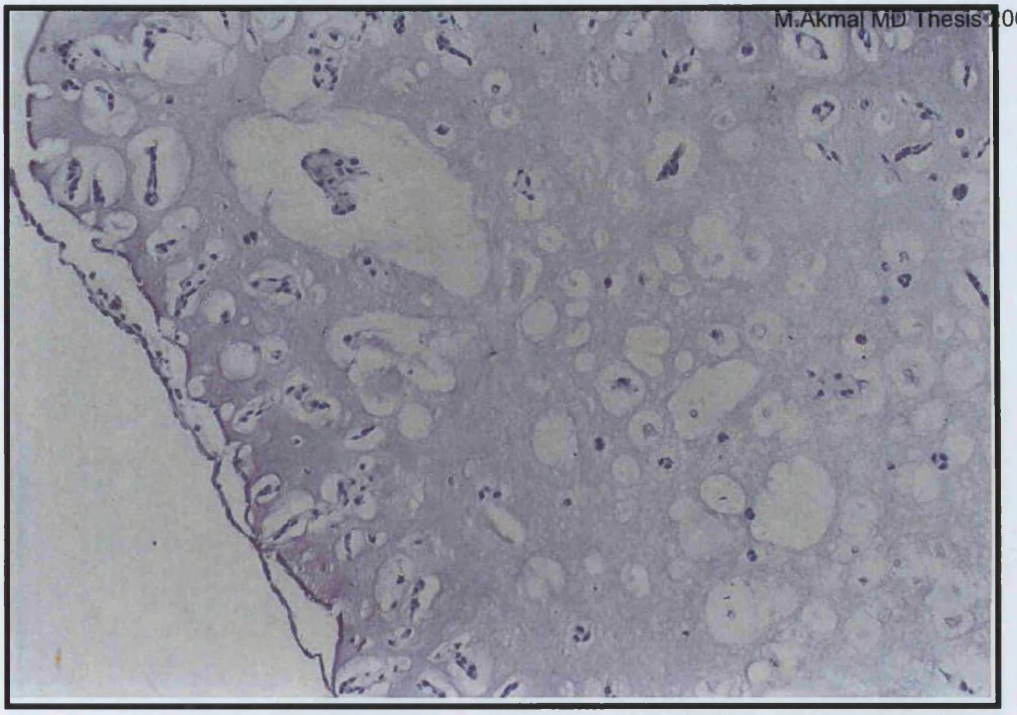


Figure 6.2.12 H&E Stain. Magn X10. Edge. Alginate bead
Day 36 culture in Static system

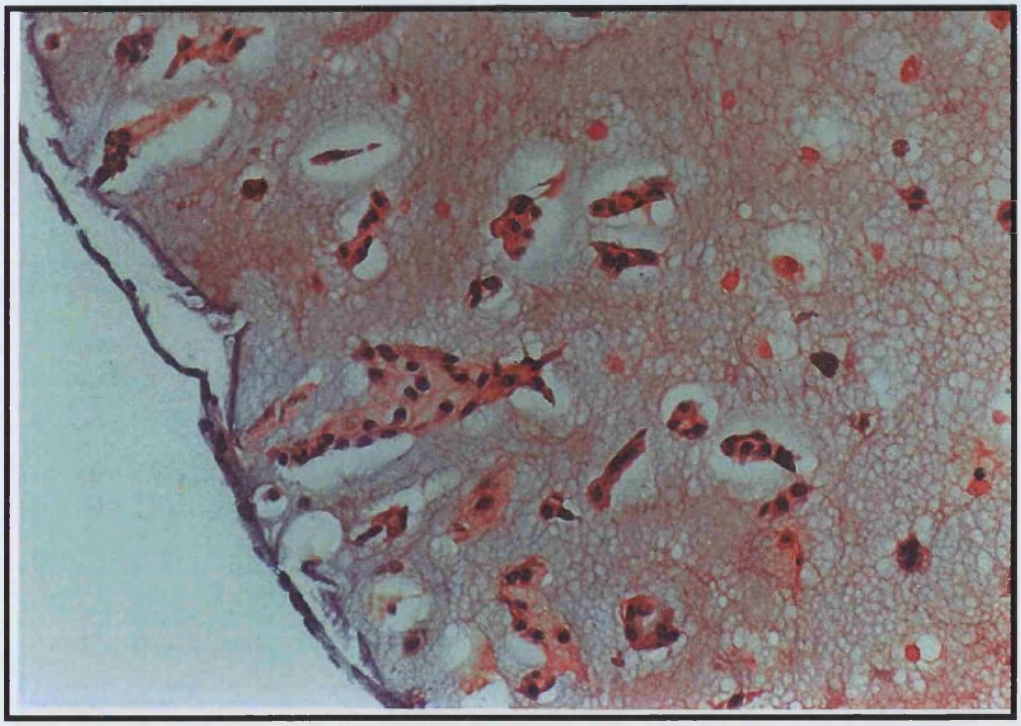


Figure 6.2.13 Safranin-O Stain. Magn X20. Centre. Alginate bead
Day 36 culture in Static system

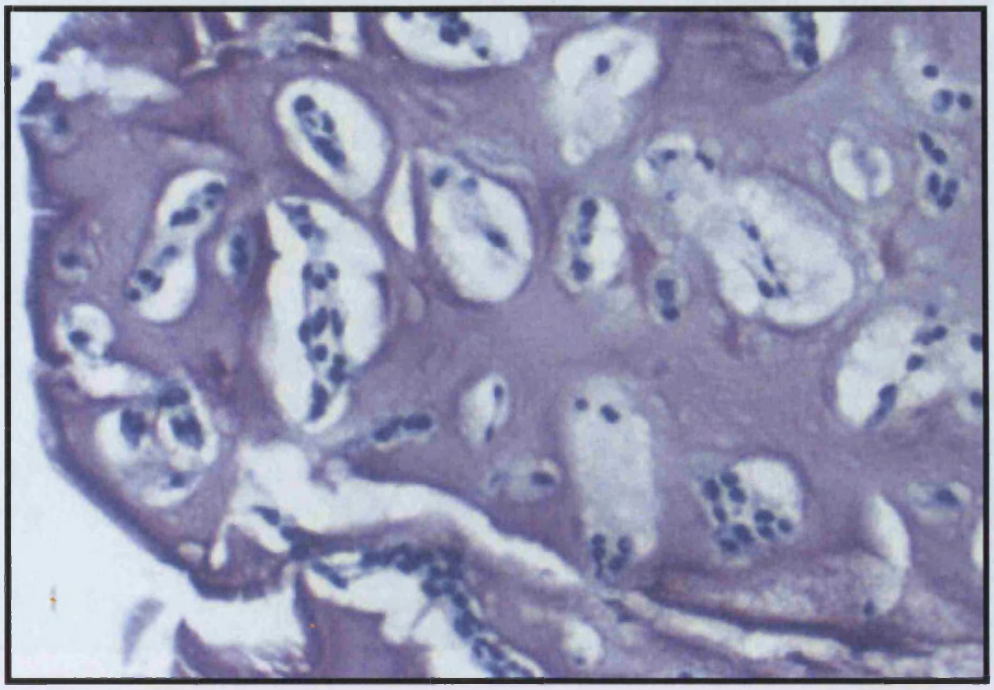


Figure 6.2.14 H&E Stain. Magn X40. Edge. Alginate bead
Day 36 culture in RWV system

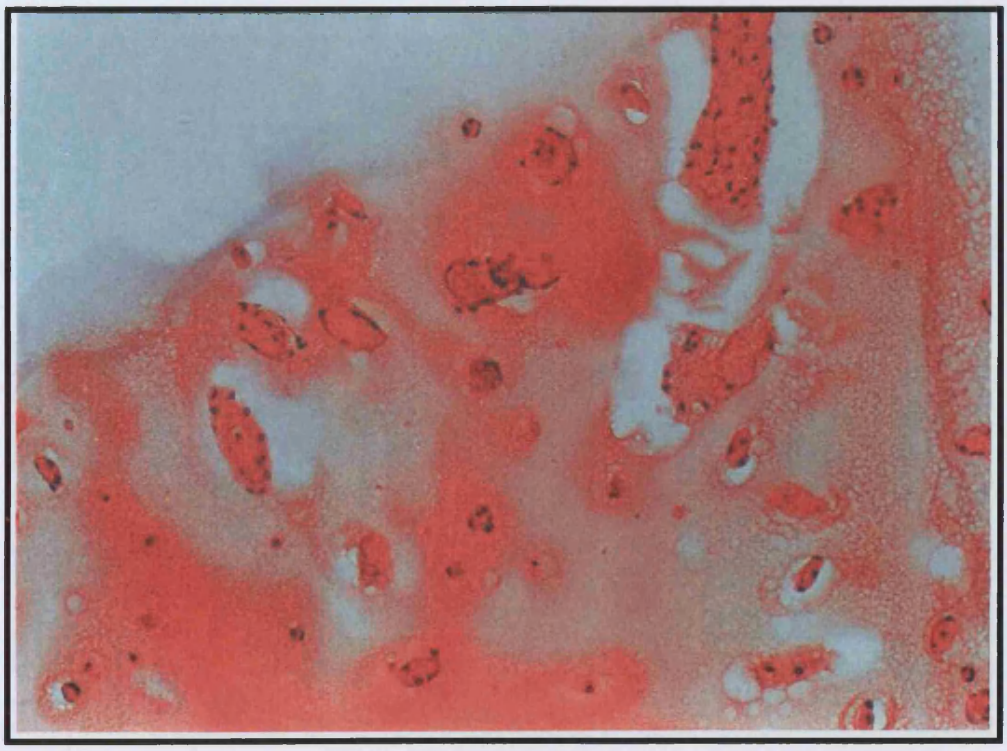


Figure 6.2.15 Safranin-O Stain. Magn X20. Centre. Alginate bead
Day 36 culture in RWV system

Figure 6.2.16 SafraninpO Stain. Magn X10. Repassaged Alginate bead Day 36 culture in RWV system

Figure 6.2.17 SafraninpO Stain. Magn X40. Repassaged Alginate bead Day 36 culture in RWV system

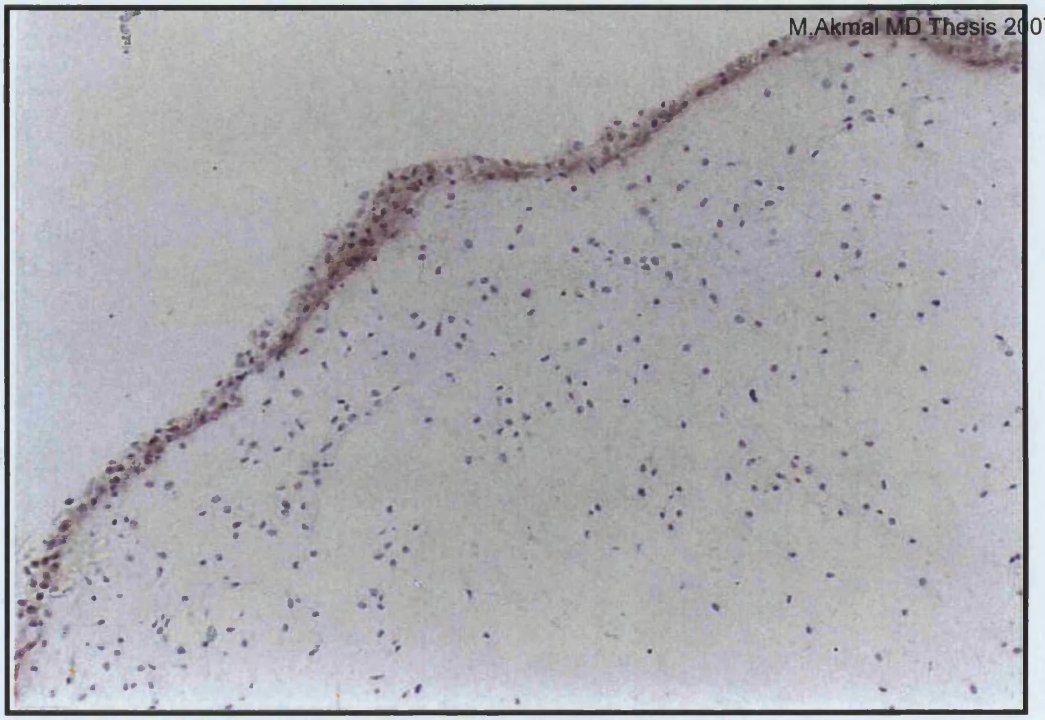


Figure 6.2.18 Collagen Type I Immunolabelling. Magn X10.
Alginate bead construct cultured in the Static system for 36 days.

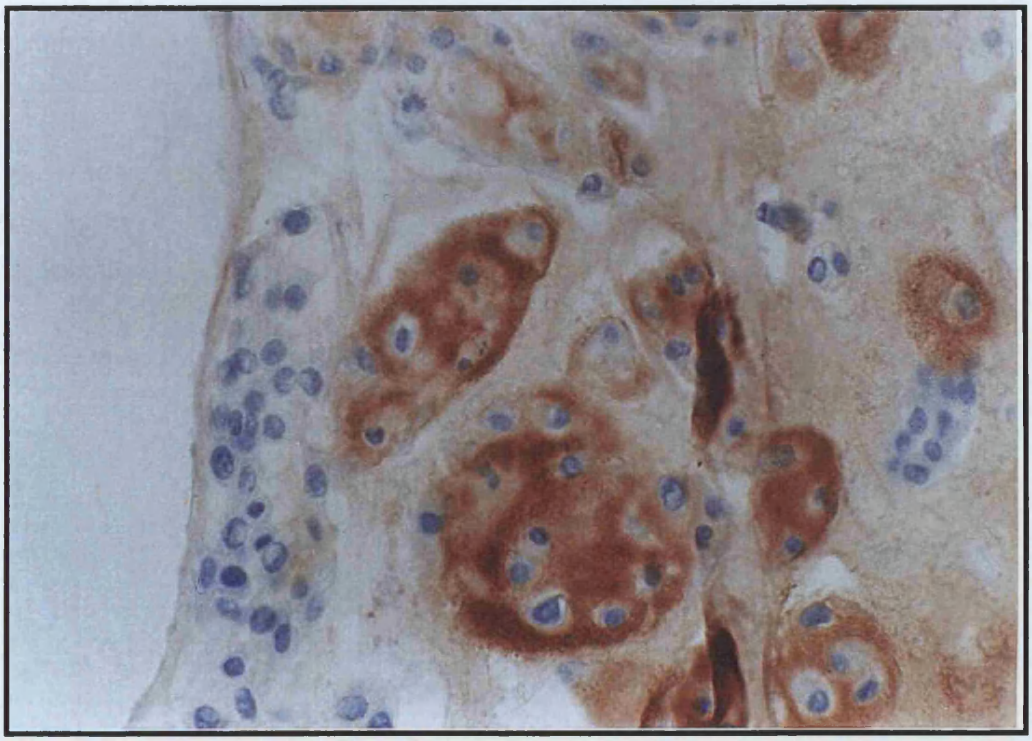


Figure 6.2.19 Coll Type II Immunolabelling. Magn X40. Edge.
Alginate bead Day 36 culture in RWV system

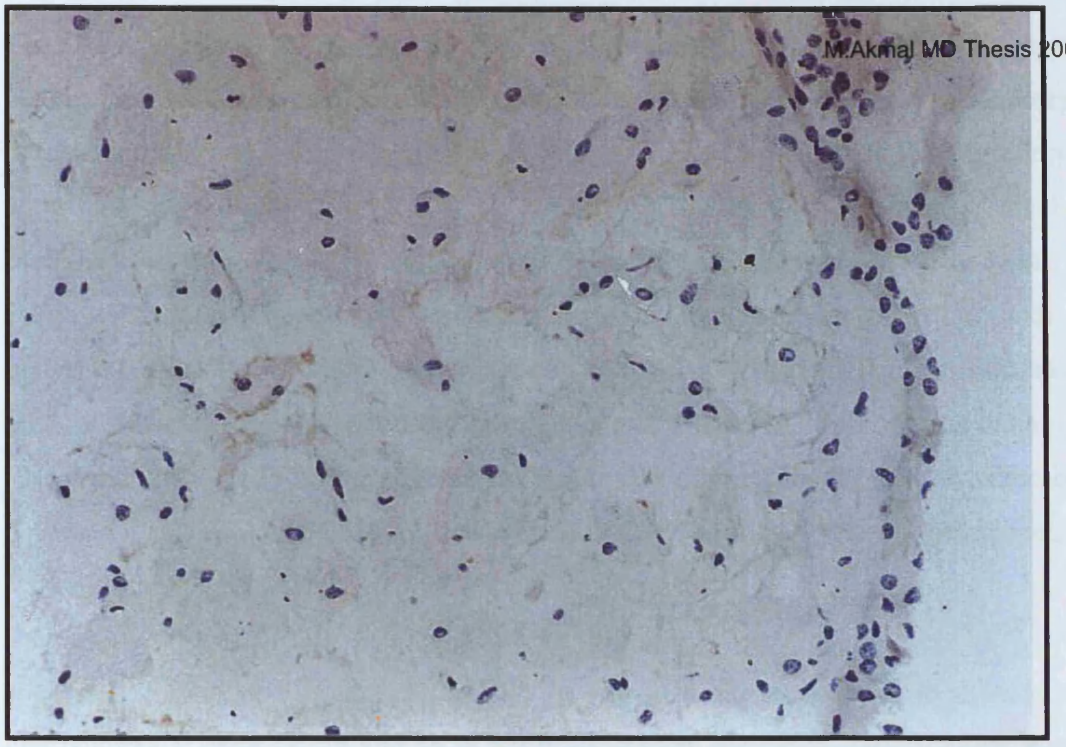


Figure 6.2.20 Ki-67 Immunolabelling. Magn X20.
Alginate bead Day 29 culture in Static system

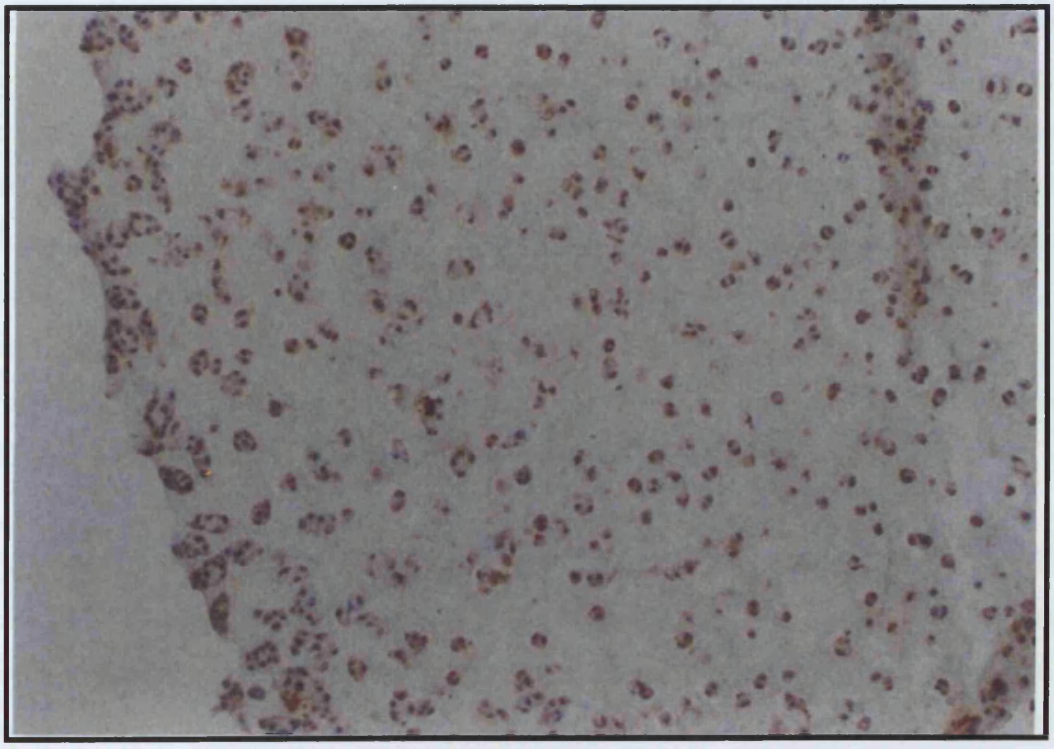


Figure 6.2.21 Ki-67 Immunolabelling. Magn X10.
Alginate bead construct cultured in the RWV system for 29 days.

Figures 6.2.2, 6.2.6, 6.2.7 & 6.2.12 show alginate bead constructs at days 21, 29 and 36 in the static culture system stained with H&E. There are large clumps of chondrocytes within the bead and the size of the clumps are much larger near the periphery as compared to the central regions. Cell clusters of 14 or more are seen at the bead periphery, whereas few small cell clusters of 3-4 are seen at the centre of the bead. The Safranin-O stains demonstrate an abundance of extracellular matrix in both the pericellular and territorial regions (*figures 6.2.3 & 6.2.13*). Higher magnification reveals a distinct demarcation between the different zones of matrix. More intense staining is seen around the cells at the edge of the bead. By contrast, at the centre of the bead Safranin-O staining was weak and present mainly localised within the pericellular region of the chondrocytes.

Figures 6.2.1, 6.2.4, 6.2.5 & 6.2.14 show the response of chondrocytes in alginate bead cell cultures at days 21, 29 and 36 in the RWV system. The H&E sections demonstrate that the cells have remained viable throughout the entire construct and have maintained their rounded morphology. Peripherally, the bead is highly saturated with chondrocytes. A large number of cells also occupy the centre of the bead, although this is less than at the periphery. The cells in the centre of the bead appear uniformly distributed and appear healthy. Cells exist in large clusters throughout the construct, with clusters of up to 50 cells seen around the edge of the bead and cell clusters as high as 30 cells at the centre (*figures 6.2.4 & 6.2.5*). *Figures 6.2.8, 6.2.9 & 6.2.15* show Safranin-O staining for sulphated proteoglycans. These sections highlight the considerable increase in intensity of staining in the RWV culture device compared to the static system. Intense extracellular matrix staining is seen throughout the entire construct, however, the staining is marked more intensely at the periphery. A large pericellular 'halo', lacuna or cell chondron is present around the majority of cells. The Safranin-O stain demonstrates an abundance of matrix in both the pericellular and territorial regions.

Figures 6.2.10 and 6.2.11 are collagen type II immunolabelled constructs at day 29 of culture in the static and RWV culture systems respectively. Collagen type II staining is present in both systems. Staining appears more intense at the periphery of the constructs in both the static and RWV systems, however more intense staining is seen in the RWV model. The static system section demonstrates a rapid decline in the intensity of collagen type II staining towards the centre of the bead, with poor staining present at the centre. The decline is less dramatic in the RWV system. The staining at the centre of the

beads remains intense and profuse in RWV beads. Staining at the centre appears more organised and uniform in distribution. The staining at the centre is however remains less intense than at the periphery.

Figure 6.2.18 demonstrates the absence of collagen type I staining within the bead however shows a capsule of collagen type I staining at the extreme edge of the bead at day 36 of static culture. This collagen type I capsule is absent in the RWV bioreactor beads.

Figures 6.2.20 and 6.2.21 are alginate beads at day 29 of culture immunolabelled with Ki-67, a specific stain for a cell proliferative antigen. *Figure 6.2.20* is a statically cultured specimen that demonstrates intense staining at the periphery of the bead, which reduces in intensity and becomes absent towards the centre. *Figure 6.2.21* is a specimen cultured in the RWV and demonstrates more intense staining throughout the bead, with staining at the centre remaining intense and uniform.

6.3 Alginate Bead Constructs cultured in a Bioreactor containing Human chondrocytes

In this experiment, the aim was to investigate the use of alginate beads for the purpose of enhancing human chondrocyte proliferation in the RWV bioreactor. Human tissue specimens were obtained from above-knee amputated limbs from the regional tumour service at the Royal National Orthopaedic Hospital, Stanmore, UK. All knee joints were tumour free at the time of collection but it was unknown whether the knee joints had previously been exposed to radiotherapy or chemotherapy. Human tissue was obtained from a total of four patients with a mean age of 23 yrs (range 17yrs – 29yrs). The cartilage was obtained aseptically within 6 hours of tissue collection from the operating theatres. The study was approved by the relevant Local Ethics Committee.

Chondrocytes were embedded in alginate beads using the same methodology as for previous bead experiments and cultured for 14 days. The beads were the same size as the aliginate beads used for bovine experiments. The rotating wall vessel device was

compared with the static culture device. The bovine cell cultures were compared with Human cell cultures.

6.3.1 Protocol

The protocol employed was exactly the same as the protocol for the agarose bead experiment (Experiment 4).

Specimens were collected at day zero, day seven, day twelve and day nineteen time points for both biochemical and histological analysis. A further set of specimens for histology were collected at day 36.

6.3.2 Results

6.3.2.1 Biochemistry

Table 10

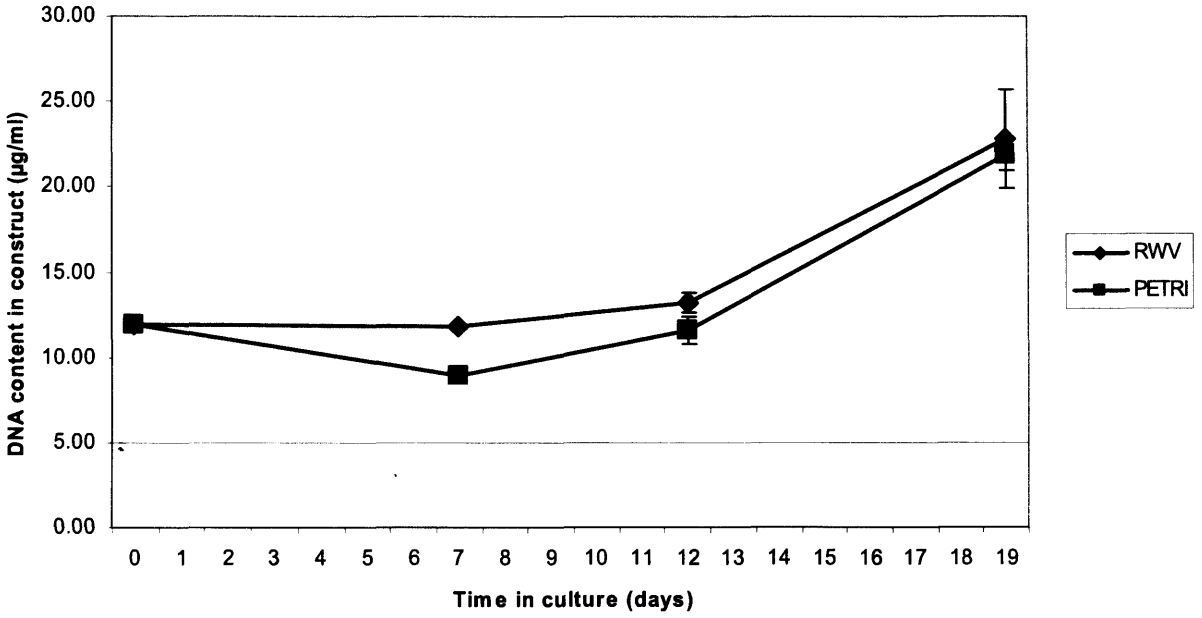
Mean DNA concentration of Alginate constructs cultured in a RWV and a Culture dish for up to 19 days

	RWV		PETRI	
Day	DNA Conc ($\mu g.ml^{-1}$)	SEM	DNA Conc ($\mu g.ml^{-1}$)	SEM
0	13.72	0.01	13.72	0.01
7	17.49	0.30	12.46	1.35
12	15.96	0.29	13.65	1.07
19	21.41	3.11	21.37	0.27

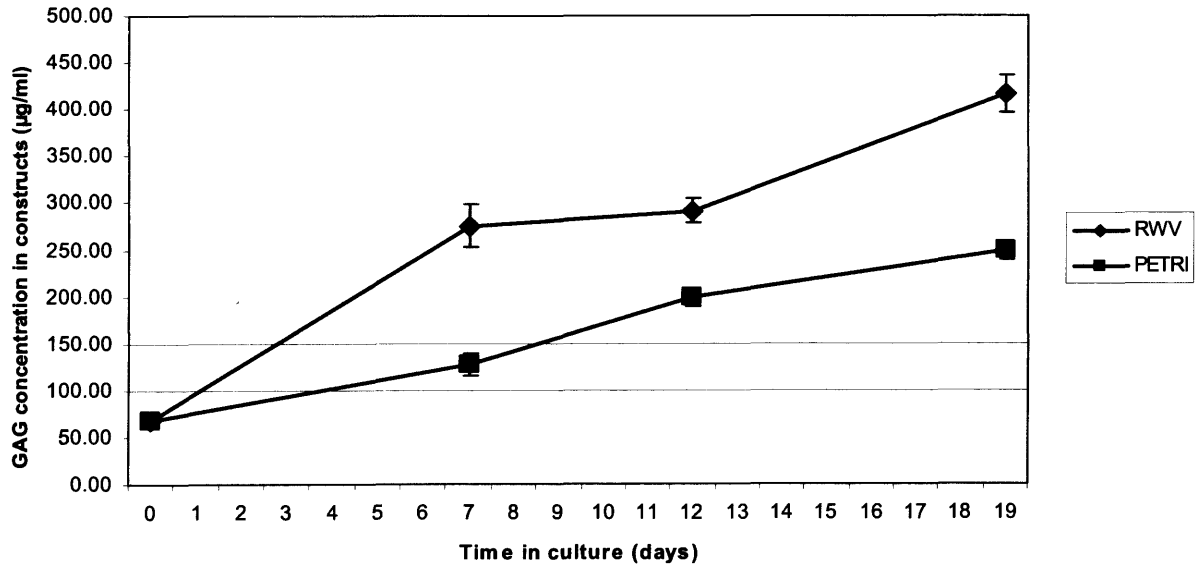
SEM = Standard Error of the Mean

Graph 17 and Table 10 show the mean DNA concentration per millilitre of alginate construct at the different time points. The initial concentration of DNA per construct was 13.72 $\mu g.ml^{-1}$ which corresponds to a cell number of 2.04 million (13.72 $x10^{-3}$ / 6.7

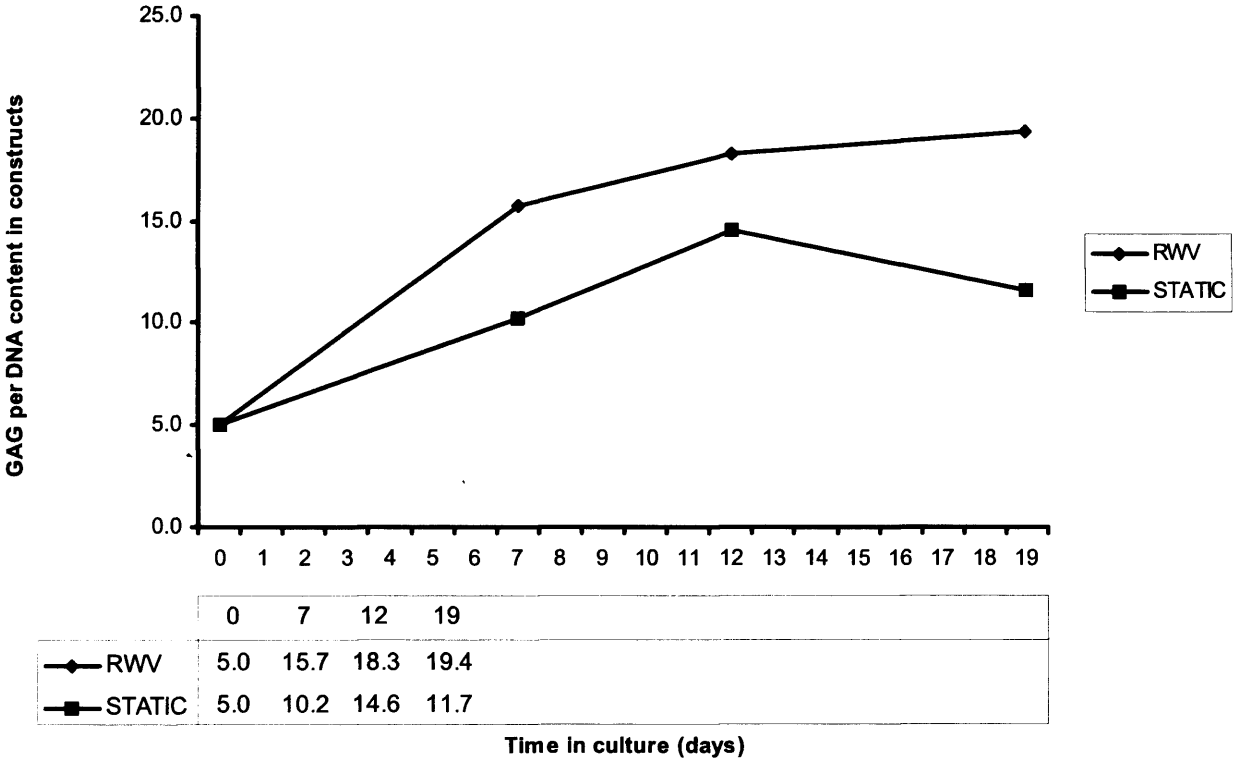
GRAPH 17. DNA content of Human chondrocyte seeded alginate bead cultured for up to 19 days in a Dynamic Rotating Wall Vessel bioreactor and a Static Petri dish system (5 beads)



GRAPH 18. GAG content of Human chondrocyte seeded alginate bead constructs cultured for up to 19 days in a rotating wall vessel bioreactor and a Petri dish (5 beads).



GRAPH 19. GAG per DNA content of Human chondrocyte seeded alginate bead cultured for up to 19 days in a Dynamic Rotating Wall Vessel bioreactor and a Static Petri dish system (5 beads)



	0	7	12	19
RWV	5.0	15.7	18.3	19.4
STATIC	5.0	10.2	14.6	11.7

$x10^{-9}$) cells per ml of construct as each chondrocyte contains approximately 6.7 x 10^{-9} $\mu g.ml^{-1}$ DNA.

Between day zero and day twelve, there was very littlel increase in overall DNA conecentration to a value of 15.96 $\mu g.ml^{-1}$ in the RWV and 13.65 $mg.ml^{-1}$ in the static device. This represents a 16% increase in the RWV and only 3 % increase in the static system. The difference between the two culture systems and between time points was not significant at all these time points ($p>0.05$)

By day 19, the differences between the RWV and the static device were still not very apparent. Both systems displayed a proliferation of cell numbers in relation to the day zero levels. The increase was 56% from day zero in the RWV and 57% in the static device. There was no significant difference between the two culture systems. The difference between day zero and day 19 time points was significant ($p<0.05$)

Table 11

Mean GAG concentration of Alginate constructs cultured in a RWV and a Static culture dish for up to 19 days.

	RWV		PETRI	
Day	GAG Conc ($\mu g.ml^{-1}$)	SEM	GAG Conc ($\mu g.ml^{-1}$)	SEM
0	68.45	7.57	68.45	7.57
7	275.15	22.93	127.44	11.84
12	291.76	12.73	199.04	8.83
19	415.69	19.70	249.14	10.75

SEM = Standard Error of the Mean

Graph 18 and Table 11 show the mean GAG concentration per millilitre of alginate construct seeded with human chondrocytes at the specified time points of the experiment. The GAG concentration in the constructs at the start of the experiment was 68.5 $\mu g.ml^{-1}$ for both groups. GAG values increased steadily throughout the culture period for both culture conditions, although the rate of increase was significantly greater

in the RWV-cultured constructs. By day 19, the GAG values were 249 $\mu g.ml^{-1}$ and 416 $\mu g.mL^{-1}$ for static and RWV cultures, respectively ($p<0.05$). The GAG per DNA graph 18 reveals a consistent increase in matrix synthesised by the cells during the culture period.

6.3.2.2 Histology

Histological images are presented for human chondrocytes in alginate constructs. *Figure 6.3.1* is a specimen from the static culture system at day 7. Cells appear viable and rounded in morphology. A few cells appear to be dividing, however, the majority of cells remain singular or in pairs. In some cases flattened cells were present at the extreme periphery of the construct. *Figure 6.3.2* is a day 7 specimen from the RWV device. At this stage the cell numbers and cellular density on the slide appear greater than the static specimen. Cells appear healthy and rounded with a greater degree of cell division occurring. A greater number of cells exist in small groups of 4 cells.

Figures 6.3.3-6.3.10 show specimens in the two systems at day 14 and 21. The specimens show that chondrocytes cultured in the static conditions are dividing considerably at the edge of the bead. Towards the centre of the bead however cell division appears to reduce, evident by the reduced cell numbers and the absence of cell clusters. The chondrocytes demonstrate a large variation in size and are distributed singly or in pairs. Most cells remain viable and maintain their spherical shape at day 21 at the centre of the bead. In comparison, cells cultured in the RWV device show high cell numbers, cell density and cell division at the centre of the bead. Cell clusters of 10 are seen at the periphery as well as at the centre of the bead, although more clusters are seen at the periphery (*figures 6.3.5 & 6.3.6*). All cells exhibited a rounded morphology and the capsule of flattened cells was absent.

In the static cultures diffuse Safranin-O staining was observed within the peripheral region with a slight increase in staining intensity around the chondrocytes (*figures 6.3.3, 6.3.4 & 6.3.8*). It also demonstrates that cells at the centre of the construct are associated with only a small amount of extracellular matrix and exhibit minimal cell division. *Figures 6.3.5, 6.3.6 & 6.3.10* demonstrate culture within the RWV bioreactor. It shows enhanced Safranin-O staining at both the peripheral and central regions, compared with the static cultures.

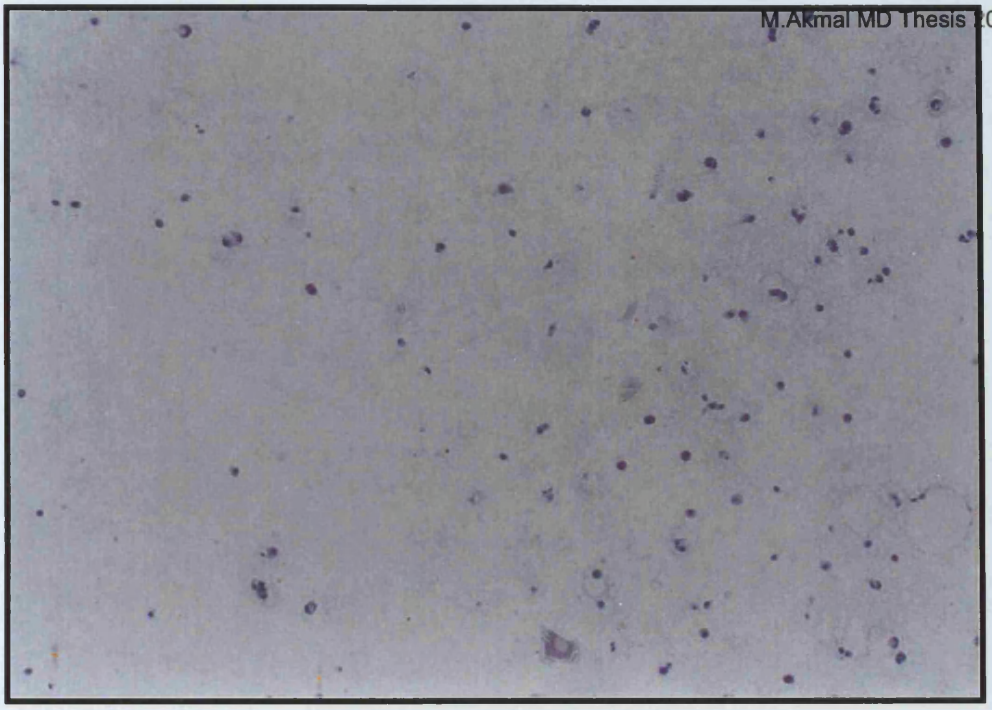


Figure 6.3.1 H&E stain. Magn 10.
Alginate bead. Human chondrocytes
Day 7 culture in Static system

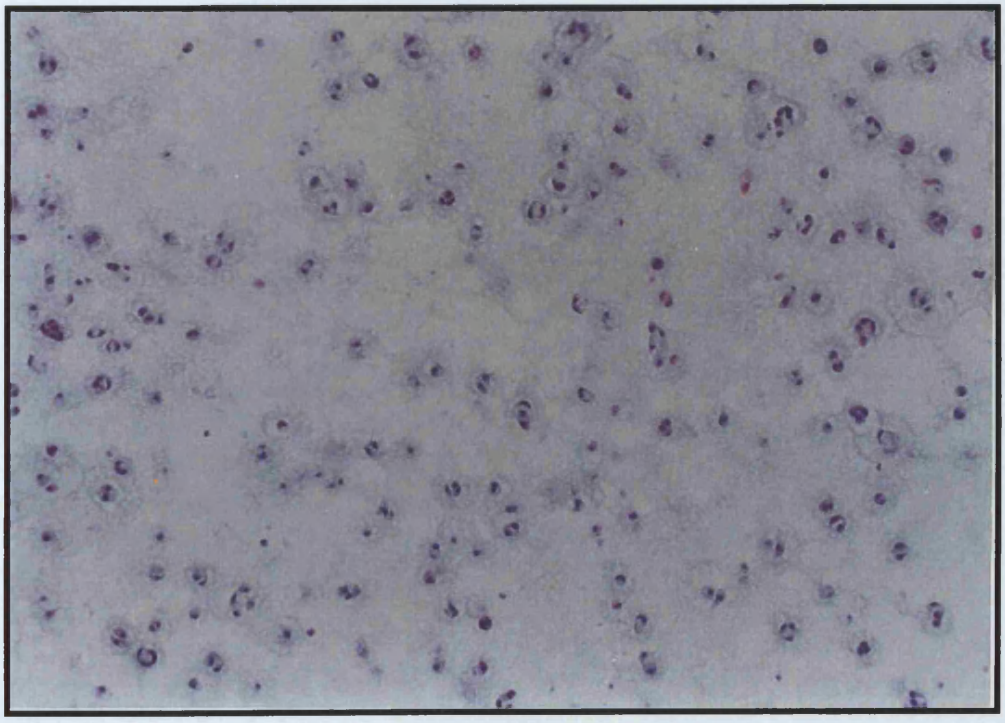


Figure 6.3.2 H&E Stain. Magn X10.
Alginate bead. Human chondrocytes
Day 7 culture in RWV system

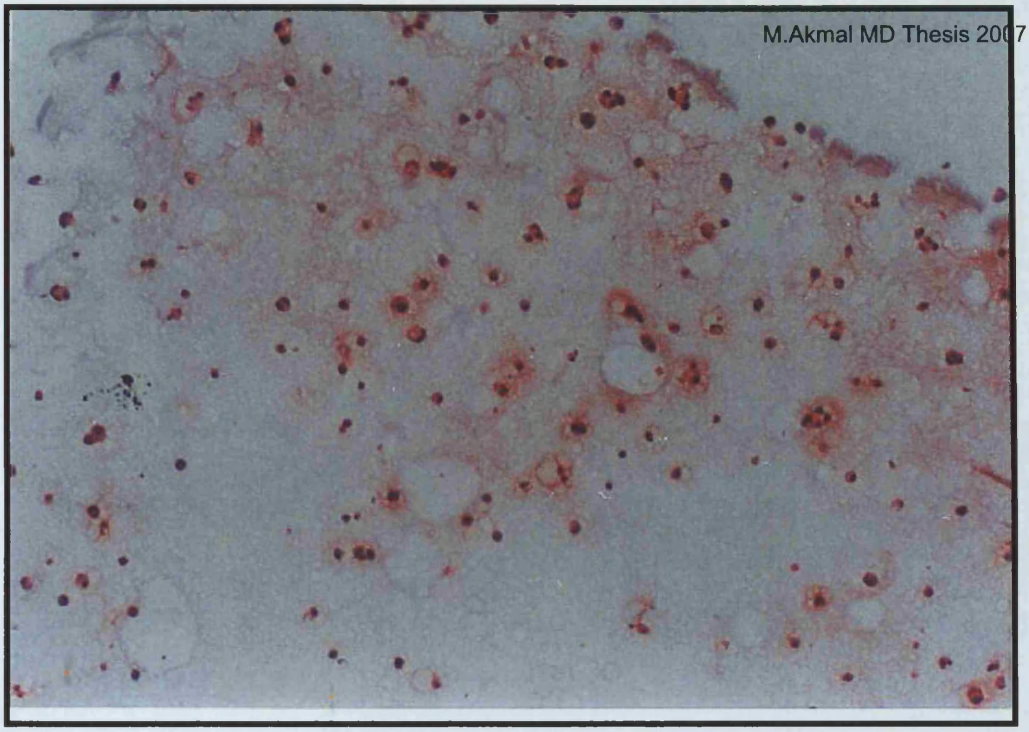


Figure 6.3.3 Safranin-O stain. Magn 10. Edge.
Alginate bead. Human chondrocytes
Day 14 culture in Static system

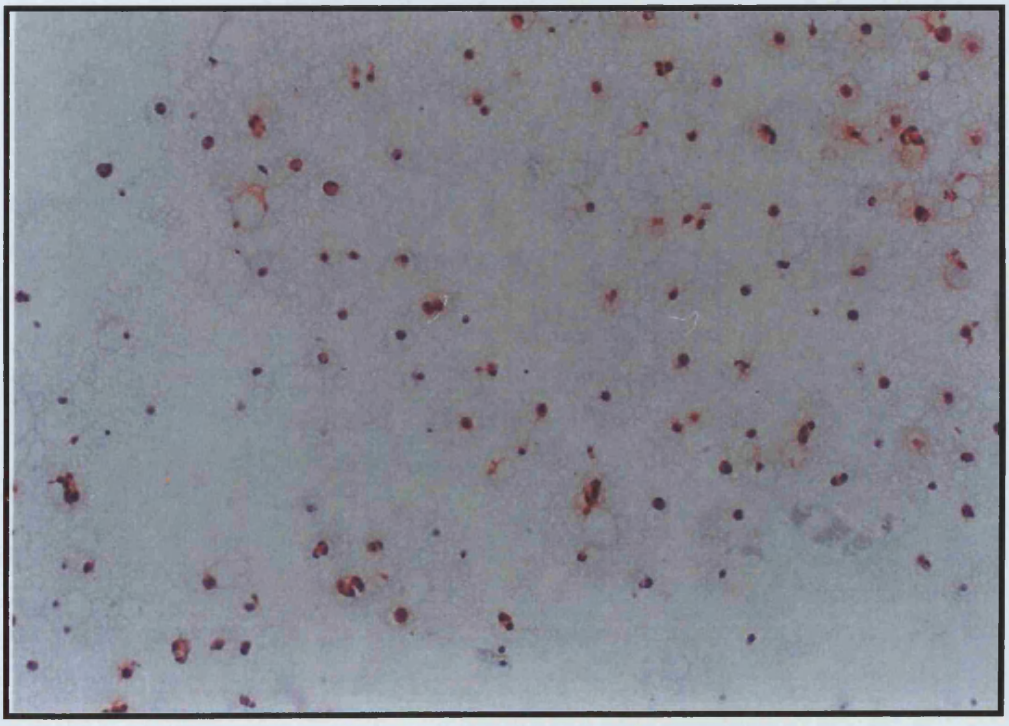


Figure 6.3.4 Safranin-O stain. Magn X20. Centre.
Alginate bead. Human chondrocytes
Day 14 culture in Static system

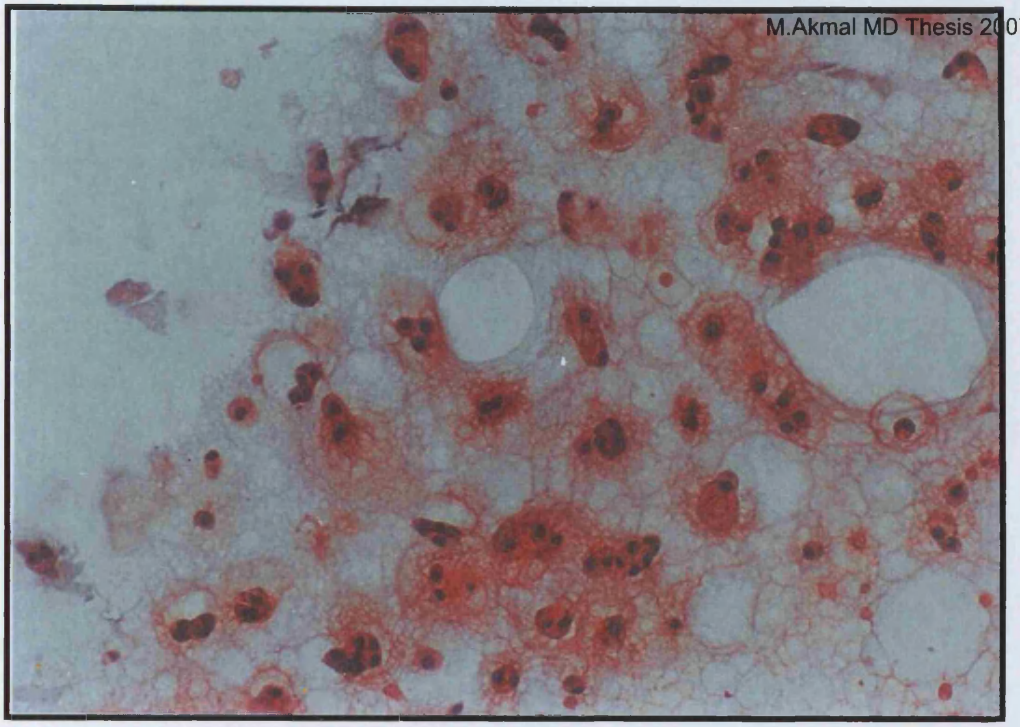


Figure 6.3.5 Safranin-O Stain. Magn X20. Edge.
Alginate bead. Human chondrocytes
Day 14 culture in RVW system

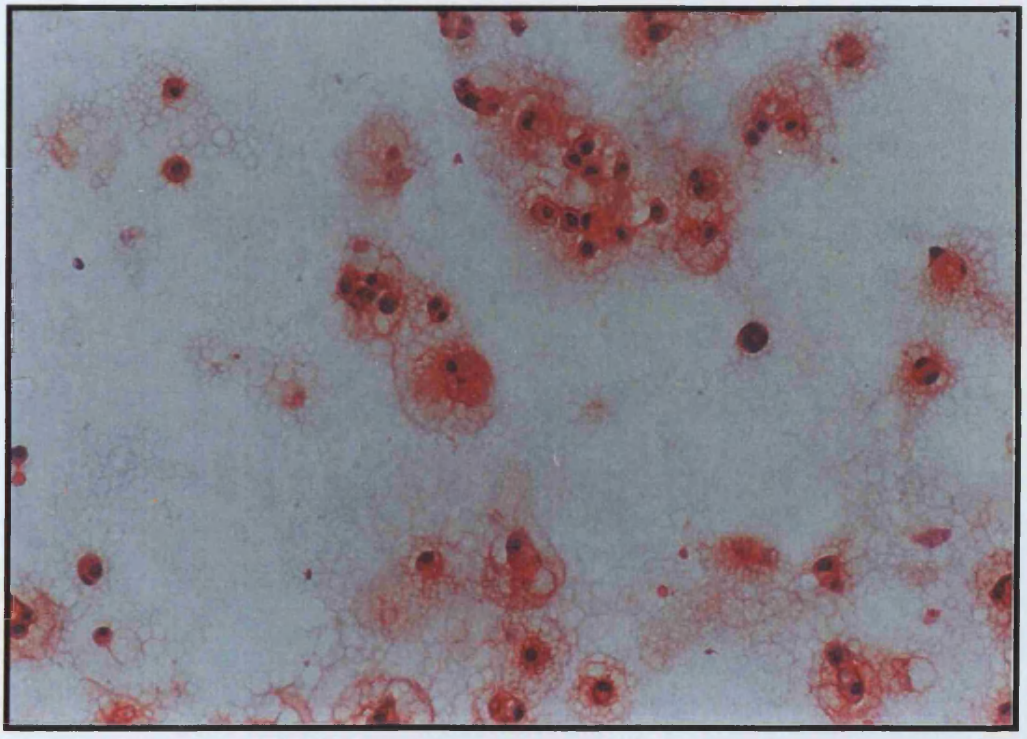


Figure 6.3.6 Safranin-O. Magn X20. Centre.
Alginate bead. Human chondrocytes
Day 14 culture in RWV system

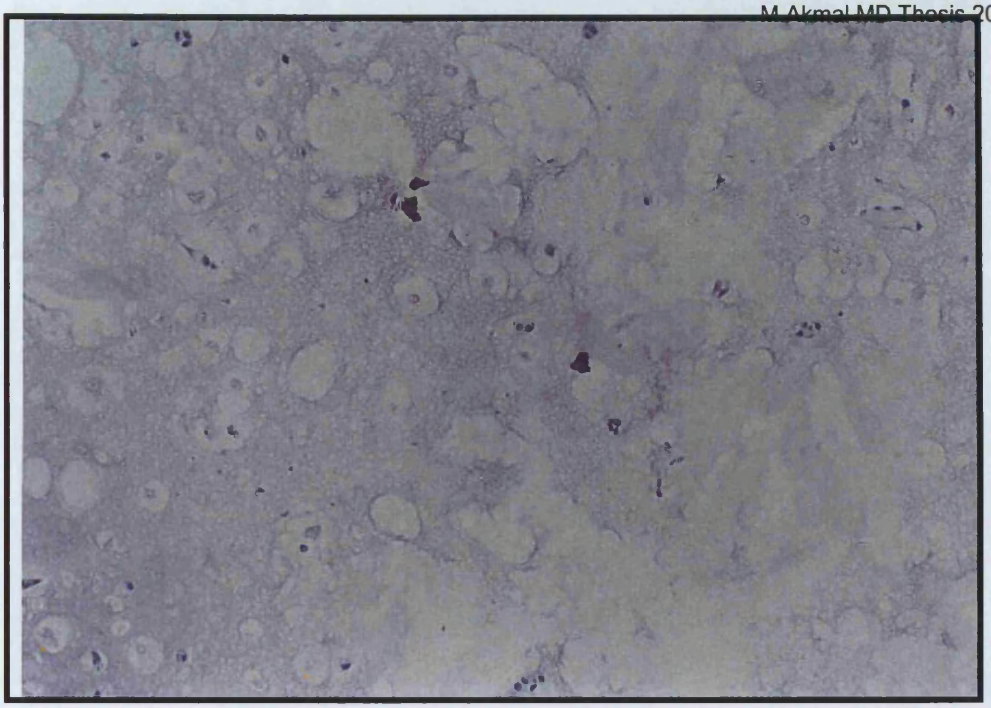


Figure 6.3.7 H&E Stain. Magn X20. Alginate bead. Human chondrocytes. Day 21 culture in Static system

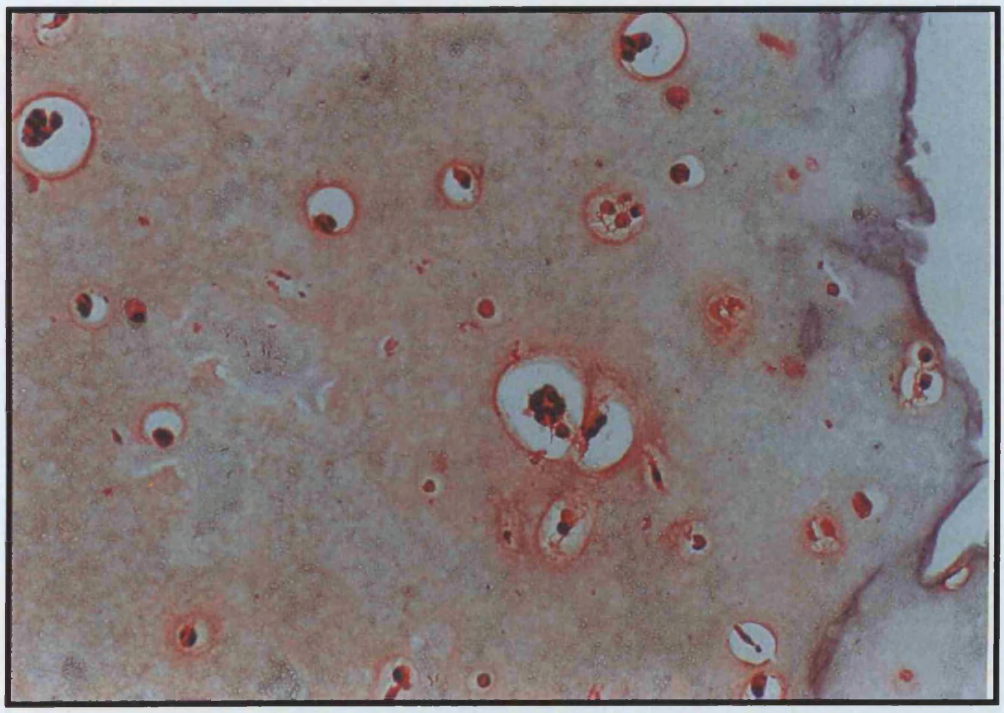


Figure 6.3.8 Safranin-O stain. Magn X20. Alginate bead. Human chondrocytes. Day 21 culture in Static system

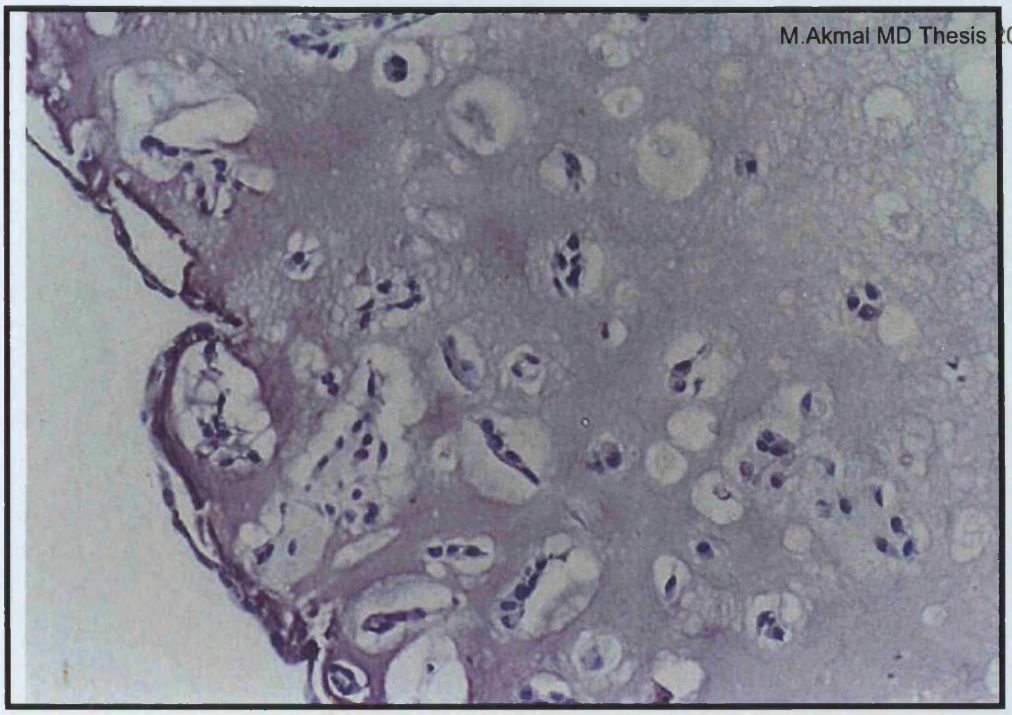


Figure 6.3.9 H&E Stain. Magn X20. Alginate bead. Human chondrocytes. Day 21 culture in RWV system

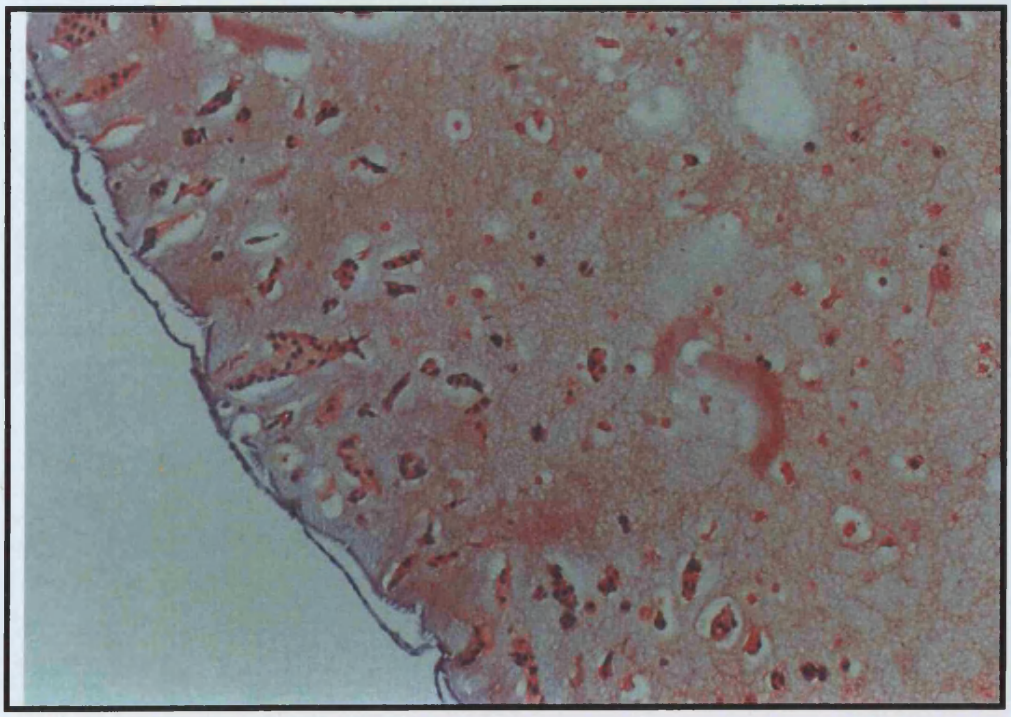


Figure 6.3.10 Safranin-O stain. Magn X20. Alginate bead. Human chondrocytes. Day 21 culture in RWV system

Figure 6.3.11 Collagen type I immunolabelling. Magn X20. Edge.
Alginate bead. Human chondrocytes
Day 14 culture in Static system

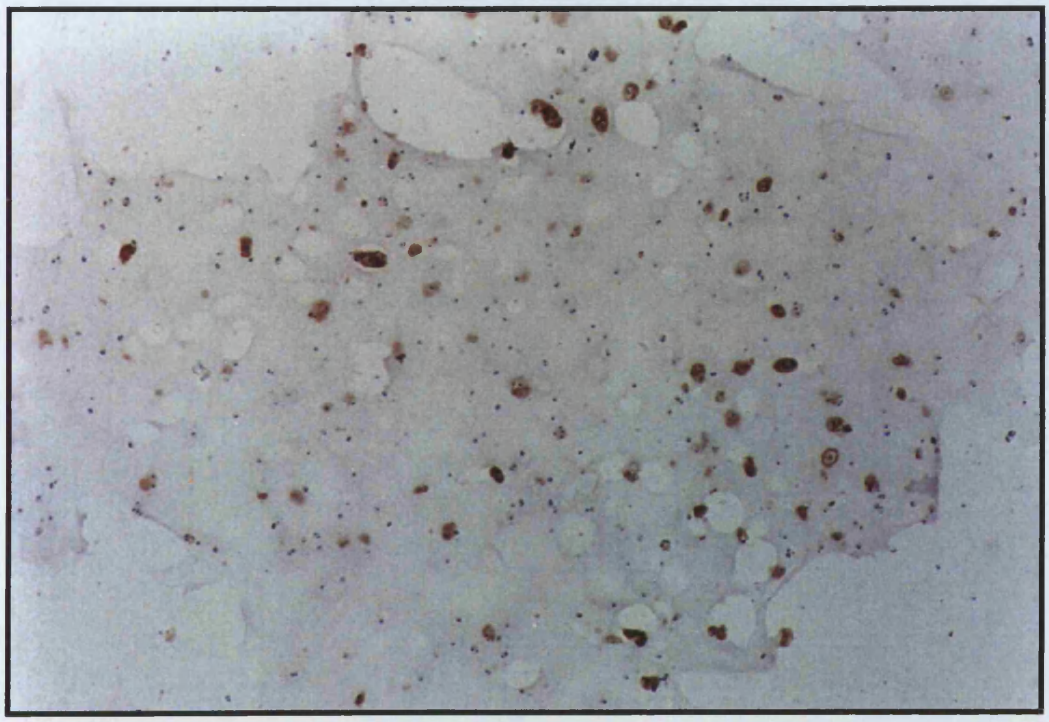


Figure 6.3.12 Collagen type II immunolabelling. Magn X20. Edge.
Alginate bead. Human chondrocytes
Day 14 culture in Static system

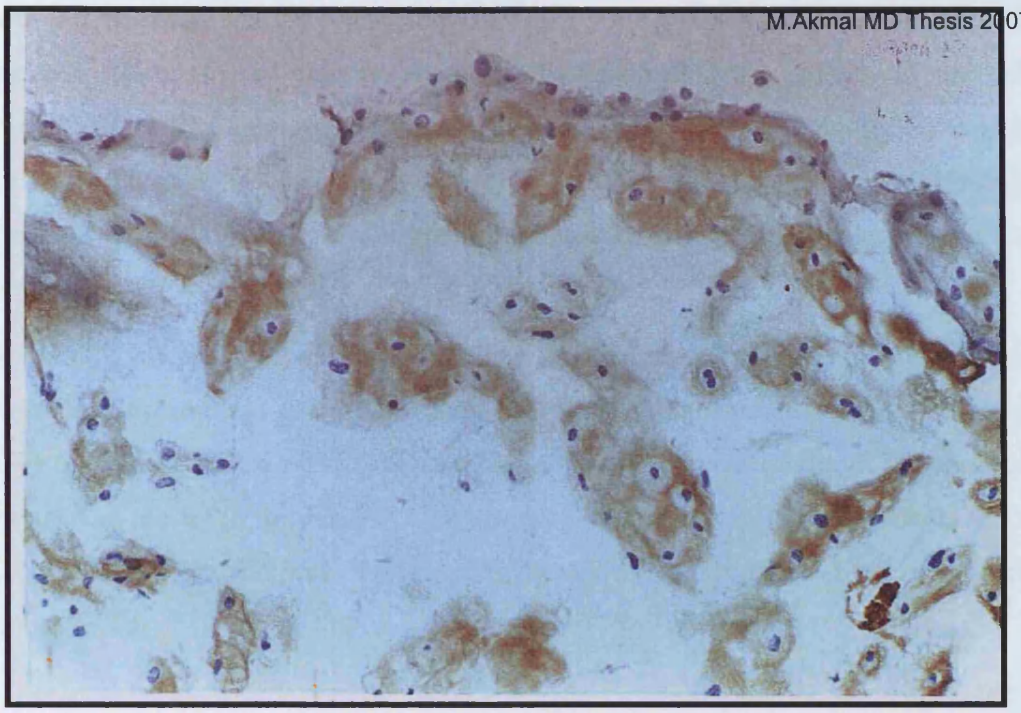


Figure 6.3.13 Collagen type II immunolabelling. Magn X20. Edge. Alginate bead. Human chondrocytes
Day 14 culture in RWV system

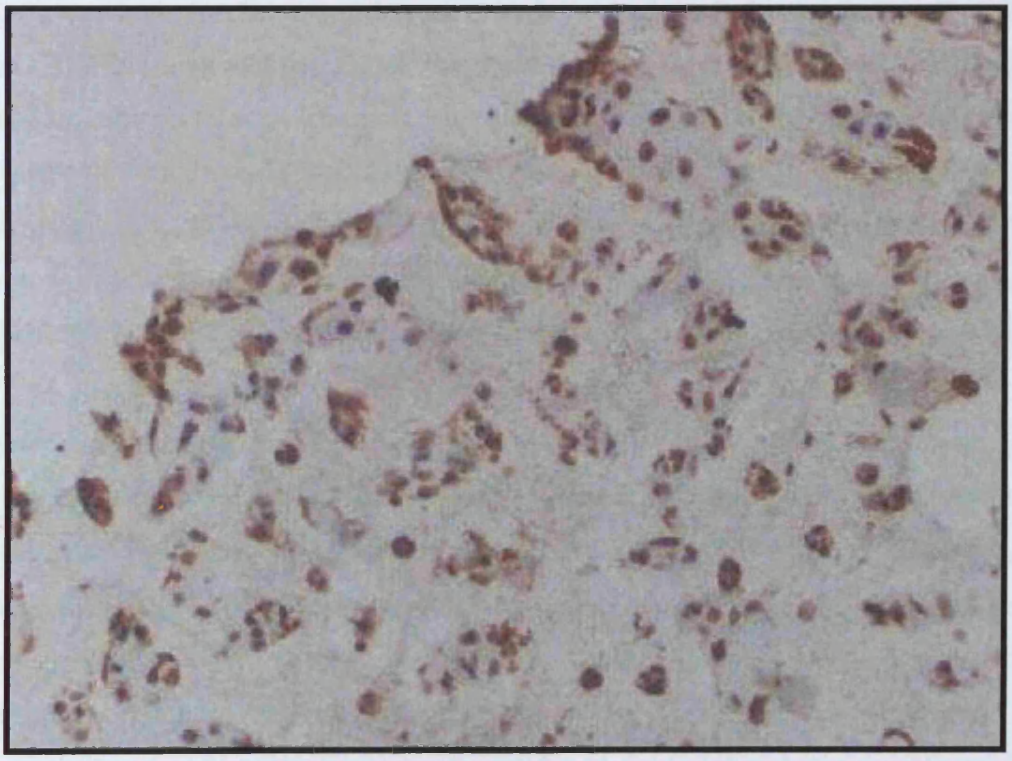


Figure 6.3.14 Chondroitin-6-Sulphate immunolabelling. Magn X20. Edge. Alginate bead. Human chondrocytes
Day 14 culture in RWV system

Figure 6.3.12 and 6.3.13 illustrate collagen type II immunolocation within the constructs. Chondrocytes express type II collagen around the surface of the cells, forming brown halos. In static cultures human chondrocytes stained poorly for collagen type II, while staining was more intense and widely distributed within constructs cultured in the RWV bioreactor. A greater quantity of staining is seen at the periphery of the beads.

Type I collagen was expressed only by cells with a flattened morphology, which formed a capsule surrounding human chondrocyte-seeded constructs cultured in static conditions (*Figure 6.3.11*). No type I collagen expression was observed for constructs maintained in the RWV bioreactor. Chondrotin-6-Sulphate staining was detected in the RWV culture system and was distributed relatively uniformly throughout the construct (*figure 6.3.14*).

6.4 Conclusion

Alginate constructs have been shown to offer an attractive strategy for tissue engineering, both for the expansion of articular chondrocytes and for use as scaffold materials. High cell densities and the material properties associated with alginate impose limitations to mass transport, and therefore dynamic devices, such as the RWV bioreactor that improve diffusional gradients may be used to enhance mass transport of nutrients to cells. The aim of these experiments was to test the hypothesis that culture within the RWV bioreactor enhances proliferation and cartilage-specific matrix synthesis by bovine and human chondrocytes seeded in alginate constructs.

Alginate constructs were cultured for up to 36 days within the RWV bioreactor and compared with a conventional static culture system. The influence of culture condition was assessed by determining total DNA and GAG content. In addition histology and immunolocalisation techniques were used to determine the spatial localisation of elaborated extracellular matrix and cells within the three-dimensional constructs and to detect the presence of phenotypic markers of chondrocytes (collagen II), fibroblast/fibrochondrocytes (collagen I), sulphated GAG (chondrotin-6-sulphate) and proliferative antigens (Ki-67).

The DNA content within the constructs at a particular time in culture is determined by the initial seeding density of cells, DNA synthesis and DNA degradation during necrosis and/or apoptosis. For bovine cells, DNA levels increased during the culture period, suggesting that cell proliferation is occurring. These findings are confirmed by histological analysis, which revealed the presence of clusters of cells formed by cell proliferation. The use of a RWV bioreactor for culturing chondrocytes results in significantly higher levels of DNA within constructs containing bovine cells (tables 6 & 8), compared to static culture techniques, suggesting an enhancement of cell proliferation within the bioreactor. The histological data support these findings; the largest cell clusters being present within constructs maintained in the RWV. By contrast, culture within the RWV did not induce marked alteration in DNA content for human chondrocyte-seeded constructs when compared to static culture (table 10).

During the first 7 days in culture, DNA levels dropped in the human chondrocyte-seeded constructs cultured in static conditions from 13.72mg.ml^{-1} to 12.46mg.ml^{-1}. This phenomenon presumably reflects DNA degradation associated with cell death within the centre of the construct. Culture within the RWV bioreactor may reduce this effect, as DNA levels remained relatively stable throughout the initial 12 days of culture. Between days 12 and 19, cell proliferation resulted in an increase in the DNA content for constructs cultured in both conditions, resulting in the formation of small cell clusters in the peripheral region of the construct.

Histological analysis also revealed the presence of a capsule of flattened cells surrounding constructs in both bovine and human cells when cultured under static conditions. The capsule was absent when constructs were cultured in the RWV. This capsule is presumably formed by the release of cells from the extreme periphery of the construct, which attach, flatten and subsequently proliferate to form a multilayer. Thus, while DNA values may be similar in human chondrocyte seeded constructs cultured in both conditions, the increases in DNA values within the RWV are exclusively related to the formation of cell clusters within the constructs rather than the formation of a capsule.

The difference in response between the bovine and human chondrocytes may reflect an inherent difference in proliferative capacity of human cells compared to other species. Mitogenic or cell survival factors released in an autocrine or paracrine fashion could

play a role in controlling cellular function within bovine seeded constructs compared to human seeded constructs.

The level of sulphated GAG within alginate constructs was measured as an indicator of chondrocyte-mediated elaboration of a proteoglycan-rich matrix. The GAG content recorded in human chondrocyte-seeded constructs was considerably lower than that expressed by the bovine cells in both culture systems (tables 7, 9 & 11).

The present data demonstrates that GAG synthesis was enhanced by culture within the RWV bioreactor compared to static conditions, for both bovine and human chondrocytes. A greater insight into the nature of the enhancement of GAG synthesis was provided by histological analysis using Safranin-O staining. The sections indicated an increase in staining in all regions of constructs cultured in the RWV compared to static culture, findings which were similar for constructs seeded with either bovine or human chondrocytes.

However, the increase in staining intensity appeared similar throughout the construct and therefore, the heterogeneity in matrix elaboration between the peripheral and central regions of the construct was maintained. It appears, therefore, that the alterations in the hydrodynamic environment provided by maintenance within the RWV bioreactor are sufficient to enhance mass transport within the constructs, predominantly by convective mechanisms. However, this process is not able to overcome the fundamental nutrient gradients associated with three-dimensional alginate constructs, which lead to a heterogeneous metabolic response and subsequent matrix elaboration. Moreover, greater utilisation of nutrients by cells at the periphery of the construct may further increase the heterogeneity within the RWV bioreactor compared to static culture conditions.

The immunohistochemical results demonstrate that chondrocytic phenotype was maintained in the three-dimensional scaffold constructs as indicated by the presence of collagen type II within constructs. Collagen types II in conjunction with aggrecan (sulphated proteoglycan), both specific markers for chondrocyte activity, have an important role in defining the mechanical integrity of articular cartilage. The enhanced staining for collagen type II (*figures 6.2.11 & 6.3.13*) in the RWV culture conditions

confirms the stimulatory potential of this system for collagen as well as proteoglycan, when compared to the static conditions (figures 6.2.10 & 6.3.12).

Significantly collagen type I, a marker for fibrocartilaginous or fibroblastic phenotype, was identified in the cellular capsule that formed around the periphery of alginate constructs containing both bovine and human cells cultured under static conditions. These finding suggest that a proportion of chondrocytes maintained in static conditions exhibited modulation to a fibroblastic phenotype, a phenomenon not exhibited in any of the specimens cultured in the RWV bioreactor.

In summary the findings presented in these alginate bead experiments demonstrate the stimulating effects of the RWV bioreactor on bovine and human chondrocytes over static culture conditions. The results support the hypothesis that culture within the RWV bioreactor enhances GAG synthesis by both bovine and human chondrocytes in alginate constructs. Moreover, cell proliferation of bovine chondrocyte-seeded constructs is enhanced in the RWV. The results demonstrate that the stimulatory effects of the RWV on the bovine chondrocytes were greater than that for human chondrocytes. In addition, the phenotype of the cells within the RWV was more stable than in static conditions with no evidence of either de-differentiation or terminal differentiation. Although the bioreactor showed heterogeneity of cell replication throughout the construct, this may not necessarily be dissimilar to normal articular cartilage which also shows a level of heterogeneity. This study also demonstrates long-term viability and sustained chondrocyte proliferative capacity without deterioration of both bovine and human chondrocytes in alginate for over a 36-day period in both static and RWV bioreactor culture systems.

Chapter VII

Discussion

Tissue engineering brings together various scientific strategies from the fields of basic science, engineering and medicine in order to improve the quality and quantity of in-vitro cell and tissue culture. Despite the limitations and challenges in terms of collaboration between various scientific disciplines, many in the academic community have commenced advanced approaches to address these issues. The work in this thesis demonstrates a multidisciplined scientific approach to address a specific problem and to go beyond the simple biological techniques and use abstract and innovational ideas in order to enhance in-vitro cell cultures. Although the work is on a cellular level rather than a molecular level, it does highlight the importance of maintaining a progressive attitude rather than stagnating at a single step and losing the wood from the trees.

The goal of most tissue engineering studies is to develop three-dimensional tissue constructs *in vitro* with the same biochemical, structural and mechanical characteristics as the natural tissue or organ. So far, this goal has not been fully realised for articular cartilage. Articular cartilage is indeed a heterogeneous structure but its strength lies in the organisation of its individual components. Like an army formation during battle, it has a layered composition which intricately maintains the structure and allows some degree of resilience against damage. The simple ability to lose hydration and regain hydration under loading and unloading conditions is one of its many characteristics that may appear simple and primitive, but are in actual fact due to complex mechanisms related to osmotic pressure gradients, crosslinking collagens and hydrophilic biochemical structure of proteoglycans. The cells themselves are responsible for the intricate structure and composition of the matrix and thereby its heterogenous properties. Indeed, cells from different regions have differing characteristics and biological functions (Hu & Athanasiou 2006).

Being an avascular tissue, adult cartilage does not have ready access to the nutrients, growth factors, oxygen supply and waste removal functions of the blood stream. It has therefore been stated and observed that cartilage has very limited capacity for self-repair within the body. However, one can observe the changes in cellular and matrix

morphology in areas adjacent to an insult both immediately and after many years (Benton, Cheng, & MacDonald 1996;Inoue & Glimcher 1982;Walker et al. 2000). There is therefore ample evidence of an attempted healing response in articular cartilage and therefore the "non-healing" status may not be completely valid. Indeed like any mature tissue, cartilage in-vivo may not regenerate after significant trauma that leads to major compositional and structural disorganisation, but it is far from proven that chondrocytes are not able to incite local repair by generating new matrix and digesting disused components thereby allowing repair to some small degree. In fact, the work in this thesis demonstrates the potential of chondrocytes to produce new matrix with a composition similar to that of hyaline cartilage. The structural organisation of the neo-cartilage on initial observation seems to be random. However, between experiments there are indeed some consistent similarities. The arrangement and configuration of the cell clumps implies mechanical influences at work. In static conditions, the spindle shapes are not produced and the cells at the surface do not take on a spheroidal shape wherae in dynamic cultures, a specific morphology is displayed based on mechanical influences. On observing the differing behaviour of the cells in terms of replication, matrix production, maintenance of phenotype and distribution, it is quite conceivable that cellular and mechanical influences both determine the nature of cartilage and that there is a real possibility of reproducing articular cartilage in-vitro by exposing the growing cells and matrix to correct external influences.

Accordingly, new approaches aimed at improving the properties of tissue-engineered cartilage are being developed. This thesis has shown that a key factor affecting the success of articular cartilage tissue culture in the laboratory is whether culture conditions appropriate for cell differentiation and rapid ECM synthesis can be provided in-vitro. Several aspects of the physical culture environment, such as mixing, hydrodynamic regime, mechanical pressure and oxygen transfer have been shown to play important roles in cartilage growth. Because these conditions are relatively easily controlled and monitored in bioreactors, it is thought that significant improvements in the quality of tissue-engineered cartilage may be obtained using this technology (Bueno, Bilgen, & Barabino 2005;Chen et al. 2004;Hsu et al. 2006;Wimmer et al. 2004). In the RWV, the speed of rotation can be varied as well as membrane thickness and various other parameters can be tweaked in order to influence both mechanical and biological influences on the cells. Typically, in research laboratories, chondrocytes suspended in scaffolds are cultivated under static conditions but in commercial settings, dynamic

cultures are used to promote cell differentiation and the synthesis of extracellular matrix (ECM) for the purposes of increasing yields (Jeude et al. 2006;Ohashi, Mochizuki, & Suzuki 1999). A combination of the two strategies may clearly lead to benefits in creating organic human articular cartilage tissue in an in-vitro setting that requires techniques from both settings. In this thesis, I have demonstrated that cell proliferation and matrix production can be enhanced significantly whilst maintaining cell phenotype. On a larger scale, the possibility of regenerating tissue for the purposes on transplantation and maintaining and storing articular cartilage in a tissue bank is also a possibility and should be explored.

My initial experiments concluded that a cell scaffold, for the purposes of tissue engineering, should not only mimic the biocomposition of cartilage in terms of proteoglycan and collagen for successful recreation of intrinsic hydrodynamic forces, but must also reproduce the biomechanical properties of intrinsic cartilage components in order to withstand loading and shear forces that occur in a joint. The construct must maintain its strength and a configuration during the culture period and once implanted into the host defect site where potentially harsh mechanical conditions may exist it must be able to integrate biochemically and mechanically.

The variety of polymers and fabrication techniques available for cartilage tissue engineering continues to expand. Pore size, porosity, biocompatibility, shape specificity, integration with native tissue, degradation tailored to rate of neocartilage formation and cost efficiency are important factors which need consideration in the development of a scaffold (Raghunath et al. 2007). Scaffold degradation during cell culture, to leave a newly generated tissue without any remnant of the scaffold material, is an important characteristic for tissues that are well vascularised in order to prevent the remnant carrier material inciting a host tissue immune reaction and inhibiting integration of new tissue This characteristic is thought not to be so relevant for articular cartilage where vascularity does not exist. The area is regarded as immuno-privelidged and new tissue will probably never integrate fully into existing tissue despite its similarity to normal host tissue and is unlikely to incite an immune reaction. All previous histological studies have shown that there is always a well defined demarcation between new tissue and existing cartilage, no matter how well the new tissue resembles the host cartilage (Peretti et al. 2006). In the case of articular cartilage tissue engineering, traditional concepts are often neglected as they may not be fully

applicable in view of the uniqueness of articular cartilage. Host biocompatibility is considered to be less important than mechanical considerations (Gratz et al. 2006). The ability to adequately fill a deficit and withstand normal articular forces without undergoing disintegration are probably more important than trying to achieve a completely well integrated, biologically similar construct.

This view of cartilage in my opinion is misguided and very narrow in regards to the functioning of articular cartilage. Although cartilage is devoid of vascularity, the synovium that surrounds all diarthrodial joints is vascular and can react to chemical and immunological influences. The synovium may indirectly cause articular cartilage damage through inflammatory mediators (Ospelt et al. 2004). I have difficulty in viewing articular cartilage as an independent tissue devoid of influences from its surrounding external environment. In fact, cartilage, synovium and subchondral bone must be considered as a single unit with direct interaction on each. Both biological and mechanical integration is vital for longevity of any tissue engineered construct. If possible, the risk of inciting an immune response should be minimised as much as possible. The bioreactor offers a more mature construct to be developed with minimal residual scaffold material at the end of the culture period thereby minimising leakage into synovial fluid. In the bioreactor, there was cell and matrix proliferation to such a large quantity that the native alginate within the scaffold was almost completely replaced or had dissolved into the surrounding medium by day 14 of culture. The replacement of native alginate with neocartilage would have potential benefits relating to leakage or degradation of alginate from the construct causing reactive inflammation in the host synoium. The bioreactor device, by improving cell proliferation and matrix production, enables not only proliferation of natural cartilage components but also a removal of unnatural carrier materials such as alginate and this would help to minimise the risk of inciting an immune response and would enhance the ability of longer term survival.

Chondrocytes isolated from bovine articular cartilage were suspended in various three dimensional scaffolds. The preliminary experiments were conducted using a collagen sponge impregnated with alginate. The collagen sponge was intended to mimic the effects of natural collagen fibres within articular cartilage and the alginate was intended to function like the proteoglycans of cartilage. The role of these components within normal articular cartilage is complementary (Buckwalter & Mankin 1998c;Mecham &

Heuser 1990;Scott 1975). The proteoglycans have an expansile property when exposed to liquid medium of lower osmotic potential ie synovial fluid and the collagen meshwork acts to restrict over expansion of the proteoglycans by acting as a physical restriction and and thereby exerting an opposing force. The equilibrium between these forces enables cartilage to maintain its consistency and viscoelastic properties. In the collagen/alginate construct experiments, the collagen sponge although being a meshwork was loosely woven and did not withstand the expansile forces of the alginate and resulted in fragmentation and failure of the construct. The dramatic failure of the construct architecture in a dynamic environment. was caused in part by the differing properties of the two individual components and in part by the harsh environment in a roller bottle. Both alginate and collagen were tending to absorb water and expand. Alginate expansion should in theory be contained by the collagen fibrils and exert a counter force to the osmotic expansion of alginate, but this did not occur and instead of the collagen fibres providing a physical restraint against the osmotic expansile force of the proteoglycans, the collagen actually expanded and further lost its meshwork architecture. The construct material instead of becoming more turgid and mechanically resilient, began to rapidly disintegrate and lost its architecture to support the chondrocytes.

The compatibility in terms of electric charge between alginate and collagen type I as used in these experiments is unknown and may have actually been a hindrance to successful integration of the two components. Close inspection of the histological images demonstrates a separation between the alginate and collagen fibres. These "spaces" within the construct have an adverse effect on mechanical integrity and lead to further disintegration of the construct. For the purposes of tissue engineering articular cartilage, the collagen type I /alginate construct is a poor model and was therefore discarded from further study. Its fragmentation and poor mechanical integrity, particulary during roller bottle culture, would exclude its use for in-vivo implantation. It would not be able to withstand the vigors of intra-articular use and may even pose added problems due to the creation of debris. Any potential scaffold design must carefully incorporate the bifunctional aspects required to mimic articular cartilage matrix and any dynamic culture device should minimise the effects of shear and turbulence which has significant adverse effects on the scaffold.

Roller bottles are crude dynamic culture devices and although satisfactory for small cell numbers, are not able to provide sufficient gaseous exchange for culturing large cell quantities. The roller bottle experiments also highlighted the importance of devising a shear resistant construct and the need to have a dynamic culture device which allows a more controlled environment in order to maintain fragile cell cultures throughout the culture period. In the roller bottles, the three dimensional scaffolds continuously collided with the container walls and this led rapidly to the destruction of the constructs. The production of bubbles resulting from turbulence caused uncontrollable oxygenation and gaseous exchange within the bottles and made the environmental conditions too unstable and heterogenous for successful tissue culture. It is likely however that in-vivo, there would be loading and shear and therefore mechanical strength cannot be completely disregarded in construct design. Indeed recent studies provide evidence that shear and multi-axial forces can stimulate chondrocyte activity and lead to a more mature articular cartilage (Raimondi et al. 2006;Waldman et al. 2003a;Waldman et al. 2003b;Waldman et al. 2007). The exact nature of mechanical stimulation or mechanotransduction that is required to enhance chondrocyte activity remains under investigation, however there is ample evidence suggesting its importance and therefore needs to be considered but the roller bottles were clearly not providing the correct dynamic conditions.

The rotating bioreactor histology specimens demonstrated a chondrocyte configuration unlike that seen in the static culture device. The arrangement of cells in the scaffold resembled native cartilage in their spindle arrangements. It is likely that mechanical influences were responsible for this observation. The exact mechanical influences of the RWV on cells was not investigated in this project but it is known that there are centrifugal, centripetal and even shear forces at the scaffold/medium interface that may be contributing to the overall effects of the deviceacting directly on chondrocytes. The analysis of these forces was not the focus of this study. The more exaggerated response of cells around the periphery of the constructs and the alignment adopted by the cells within the cell clusters may be related to small shear forces occurring at the interface whereas the deeper cell configurations may have been due to the gravitational forces.. The bioreactor conditions were obviously more conducive to cell and matrix proliferation than the roller bottle environment. The presence of a heterogenous cell configuration is also seen in native cartilage and this together with the varying matrix morphology at the cartilage surface may be related to the function of articular cartilage.

Archer et al has shown that surface chondrocytes in articular cartilage express different collagens and this causes different matrix configuration at the surface (Archer et al. 2006;Kavanagh et al. 2006;Khan et al. 2001). It is possible that cells at the surface by virtue of greater oxygenation or by experiencing greater shear forces exhibit a different formation and produce different matrix components in response to biomechanical influences. These differing structures allow cartilage to have a resilient surface and prevent the loss of macromolecules into the synovial fluid and may also be responsible for cartilage repair at the surface.

An artificial scaffold composed of a hydrogel has suitable mechanical properties to maintain chondrocyte cultures for long periods without disintegration. But by improving mechanical strength, mass nutrient transfer is often compromised. When scaffolds are cultured in static devices, there is a significant problem with oxygen and nutrient transport into the centre of the scaffolds (Martin & Vermette 2005). The histological results from the static and large scaffold culture experiments in this thesis demonstrate the deficiency of static culture systems to allow adequate nutrient transfer and waste disposal from the centre of three dimensional scaffolds. The poor viability and lack of proliferation of cells and lack of matrix production, particularly in static cultures, at the centre of the constructs is a direct result of poor gaseous and nutrient transport. In the bioreactor however, the nutrient gradients did not pose such a significant restriction. Even cells deep within the constructs were proliferating and synthesising new matrix. One of the most significant advantages of a dynamic culture environment is the ability to replenish medium at the scaffold/medium interface and thereby maintain a large nutrient and waste product diffusion gradient ensuring successful and rapid transport of tese products through the matrix. The mass transfer of nutrients was the main limiting factor for cell growth, replication and matrix production towards the centre of the agarose discs. Agarose beads were employed successfully to overcome the limitations posed by larger discs in terms of nutrient transfer. By using smaller constructs ie beads, which are one tenth of the size of discs it was confirmed that nutrient transfer was the rate limiting factor in earlier experiments. In beads, the rate of cell proliferation and matrix production was greatly accelerated as compared to larger constructs which demonstrated cell death towards the centre

The experiments using agarose as the three dimensional scaffold in the rotating wall vessel were somewhat surprising in that firstly such a significant proliferative response of the chondrocytes was not expected and secondly the large aggregates of cells had not been previously observed in any form of static cultures. The cell clumps were far more abundant around the periphery of the constructs as compared to the central regions. In some cases, the cell clumps were attached to the outer surface of the construct by a stalk. This configuration was far more abundant in agarose bead cultures in the RWV and was not present in any of the static cultures or alginate constructs. Although the origin of these large cell clumps is difficult to identify accurately, it is likely that the initial cell seeding of the scaffolds included a few surface attached chondrocytes that were able to replicate and grow on the surface. It is also possible that cells replicating within the construct were able to somehow migrate or become ejected from the construct onto the outer surface. Active chondrocytes are known to possess cilia or tentacle like processes and unreported electron microscopy experiments demonstrate these processes very well. It is postulated that they act as mechanotransducers, but whether these outgrowths can help to allow chondrocyte movement or have more than just a sensory role is undetermined (McGlashan, Jensen, & Poole 2006). The presence of a three dimensional matrix surrounding chondrocytes appears vital for normal articular chondocyte function (Bassleer et al. 1986;Gibson, Schor, & Grant 1982). Abundant in numbers, these surface clumps did not have any surrounding matrix and were phenotypically dissimilar to chondrocytes within the scaffold. The quantity of cells free floating in the medium was not measured in these experiments but would have provided interesting results. I suspect there was a large amount of cell and matrix product in the medium and this was not accounted for in the final biochemistry results.

After a 14 day culture period, the agarose beads became packed with cells and matrix. Histological and biochemical results were very consistent in demonstrating the considerable proliferative effect of the RWV. Despite the much improved conditions in the RWV, this process was not able to overcome completely the fundamental nutrient gradients associated with three dimensional constructs, which led to a heterogeneous metabolic response and subsequent matrix elaboration. Moreover, greater utilisation of nutrients by cells at the periphery of the construct may have further increased the heterogeneity within the RWV bioreactor compared with the static culture condition.

One of the other observations noted and not reported on in this thesis is the improvement in infection rates obtained by the RWV as compared to other open dynamic culture devices such as magnetic stirrers or roller bottles. Culture infections have the potential of seriously hampering both laboratory research and more importantly clinical tissue engineering prospects. A single infection can cause the loss of multiple specimens and even multiple experiments can be ruined following a single infection in an incubator and therefore this factor alone may justify the use of an advanced culture device for the purpose of clinical cartilage tissue engineering.

Alginate is a biocompatible and biodegradable substance which has good nutrient transfer properties. The use of alginate hydrogel scaffolds for chondrocyte culture resulted in a proliferative response that was different in nature to the agarose scaffold. The cells multiplied many times and matrix production was abundant. The cells formed much larger clumps and tended to become far more packed than in the case of agarose. The differences between RWV and static culture were much more convincing. Despite the immense cellular and matrix proliferation, the chondrocyte phenotype was maintained and a hypertrophic conversion as observed in other rapidly proliferating chondrocytes was not seen (Mwale et al. 2000). This was confirmed by immunolabelling for collagen type X which was absent in the culture specimens. The alginate density was optimised by trial and error to achieve maximum cell replication and minimal loss of mechanical integrity. The RWV was able to sustain chondrocyte cultures for far longer periods without loss of phenotype as compared to agarose. In some cases the culture period was extended to 56 days without loss of phenotype. However, all cultures maintained for periods longer than 14 days displayed a slower increase in cell numbers as construct became more compact with cells. This suggests the possibility of a limitation on diffusion rates of nutrients or maybe some form of negative feedback mechanism that is inherent within cartilage and chondrocytes. Recent studies on the effects of hyaluronic acid on chondrocytes implies the presence of a hyaluronic acid, CD44 receptor, on chondrocytes that may be responsible for the feedback effects of matrix on chondrocytes (Akmal et al. 2005).

Human chondrocytes have much slower proliferation rates than cells of most other mammals. The reasons are unknown, but may represent a more terminal cell type in humans ie a more "committed" state of differentiation along the lineage tree. Human articular joints undergo highly specialised ranges of movement and the articular

surfaces are often more complex than those of bovine joints. The human cell experiments failed to demonstrate the enhancing effects of dynamic culture as seen with bovine cells. The experimental design for the human cell cultures was exactly the same as for the bovine experiments and this may have in fact been unsuitable for human cultures. The use of human serum would have been a more suitable component of the culture medium for human cells rather than fetal calf serum as used in these experiments. In addition, although every effort was made to obtain human chondrocytes from healthy patients for the experimental work, we were only able to obtain sufficient tissue from bone tumour patients and some of these patients underwent either chemotherapy or radiotherapy to the involved limb prior to surgical amputation. These treatments may have caused abnormalities in the chondrocytes to reduce their viability and/or proliferation ability. Future work should be aimed at optimising the culture conditions for human chondrocytes and a consistent source of human tissue, possibly from a cell line, without previous exposure to damaging radio or chemotherapy is essential. The ethics surrounding tissue cloning make this sort of research extremely difficult at present although we have started a study on amputated tissue from non radio or chemo exposed patients. The need for ethical committee approval and strict consenting procedures within the NHS need to be addressed prior to conducting any future projects.

Recently the rotating vessel bioreactor has been used to investigate the differences in properties between mature and immature constructs of cartilage. Immature constructs had poorer mechanical properties but they integrated better into the host site than mature constructs. Integration of immature constructs involves cell proliferation and progressive formation of cartilaginous tissue, while integration of mature construct involved only the secretion of ECM components (Obradovic et al. 2001). In these studies, the bioreactor was able to provide a controlled and regulated environment for the study of factors affecting the integration of engineered cartilage into native cartilage and adjacent tissue without the influence of systemic effects and variability that are inherent in in-vivo studies. The rotating wall vessel bioreactor is able to simulate a dynamic environment and as its speed of rotation and other factors can be carefully controlled electronically with very precise changes, it offers an opportunity to compare various hydrodynamic stimuli to culture constructs. The successful culture of chondrocytes and abundant matrix production in the RWV in these experiments suggests that the conditions within the bioreactor are suitable for chondrocyte and

matrix maintenance and may be more similar to intra-articular conditions than those that exist in static cultures. The presence of collagen type II abundant matrix, ie articular cartilage type matrix, demonstrates its ability to resemble in-vivo conditions with a far better accuracy than a static culture device. A long-term objective of cartilage tissue engineering is to produce transplantable tissues for the treatment of injured or diseased joints and for use in reconstructive surgery and clearly the RWV may offer greater potential then most other dynamic culture devices available today. However, beyond its uses for tissue engineering, it may also provide a controlled and regulated evironment for the investigation of drug effects and other stimuli on chondrocytes. This potential uses of this technology extend to a wide range of applications, including as a substitute for animals in toxicity trials, in animal-free medical research, and as a production system for therapeutic compounds and growth factors synthesised exclusively in differentiated tissues.

The work in this thesis has demonstrated the potential of using dynamic culture devices to promote and stimulate articular chondrocytes to replicate and deposit increased amounts of extracellular matrix. The increased rates of cell replication and deposition of abundant matrix is an important factor in establishing a successful tissue engineering articular cartilage project. With regard to a stable phenotype of chondrocytes and no deposition of abnormal matrix components despite the rapid proliferative nature of the cells, it is possible to now conduct in-vivo animal studies and this would be the future direction of any work. The possibility of implanting a cell seeded construct into articular cartilage defects is definitely on the horizon and the work in this thesis will be pivotal in the development of future strategies in improving cartilage tissue engineering procedures and would provide an alternative, possibly more successful method, of chondrocyte transplantation than the existing, fairly primitive, methods.

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